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THE BILE SOLUBILITY OF PNEUMOCOCCUS, WITH SPECIAL REFERENCE TO THE CHEMICAL STRUCTURE OF VARIOUS BILE-SALTS.

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METHODS have recently been described by White (1929) for the preparation of pure deoxycholic acid and cholic acid—substances which have proved satisfactory for bile-solubility tests with pneumococci. A series of derivatives of these two substances has now been prepared and tested on pneumococci, to determine the relation of lysis to the structure and properties of the bile-acids. The relative solubility of different strains of pneumococci in solutions of the sodium salts of these acids has also been investigated. The results of these tests are given in this paper.

The bile-acids tested may be divided into two classes (Table I): (*a*) uncombined bile-acids, which differ from each other in chemical structure; (*b*) choleic acids, which are addition compounds of deoxycholic acid, and differ only in the nature of the added substance.

Twenty-five strains of pneumococci were tested with each bile-acid preparation. Of these, 16 were stock strains which had been cultivated on artificial media for some months; the others were recently isolated from pathological conditions in man. All the strains were typed with sera prepared in rabbits against known type strains received from Dr. F. Griffith. Seven strains belonged to Type I, three to Type II, four to Type III, five to Group IV, and six strains were rough variants derived either from Type I or Type II strains by growth in the corresponding homologous serum according to the method described by Stryker (1916), Griffith (1923) and others.

PREPARATION OF BILE-ACIDS.

Cholic acid (m.p. 198° C.),* acetic-choleic acid (m.p. 143°–144° C.) and deoxycholic acid (m.p. 165°–169° C.) were prepared by the method of White (1929). As all the other bile-acids tested were prepared from the first two,

* The melting-points are those of the products used for the tests.

these were carefully purified and freed from all trace of higher fatty acid. In addition to the usual purification (White, 1929), the cholic acid was extracted with petroleum ether and recrystallized three times from alcohol ; the acetic-choleic acid was again boiled with xylol and recrystallized twice from glacial acetic acid. Apocholic acid (m.p. 160°–168° C.) and dehydrocholic acid (m.p. 236°–238° C.) were prepared from cholic acid, the former by the method of Boedecker (1920) and the latter by that of Hammarsten (1881), dehydro-deoxycholic acid (m.p. 185°–187° C.) from acetic-choleic acid by the method of Wieland and Sorge (1916).

The choleic acids were prepared from acetic-choleic acid on the lines suggested by Wieland and Sorge (1916), using the purest available substances. The products were purified in the following manner : cholesterol-choleic acid (m.p. 155°–157° C.), benzaldehyde-choleic acid (m.p. 155° C.), and camphor-choleic acid (m.p. 173°–175° C.) by extraction with petroleum ether ; propionic-choleic acid (m.p. 150°–160° C.), palmitic-choleic acid (m.p. 184°–185° C.), stearic-choleic acid (m.p. 186°–188° C.), oleic-choleic acid (m.p. 184°–185° C.), tributyrin-choleic acid (m.p. 155°–160° C.) and triolein-choleic acid (m.p. 184°–185° C.) by extraction with petroleum ether followed by recrystallization from alcohol ; xylol-choleic acid (m.p. 180°–182° C.) by crystallization from alcohol ; benzene-choleic acid (m.p. 161°–163° C.), acetone-choleic acid (m.p. 160°–162° C.) and ether-choleic acid (m.p. 153° C.) by recrystallization respectively from benzene, acetone and ether. All products were finely powdered and dried *in vacuo* over soda-lime at room temperature for 48 hours.

PREPARATION OF BILE-SALT SOLUTIONS.

Ten per cent. solutions of the bile-salts were prepared by weighing out a definite quantity of bile-acid in a sterile bottle, adding two drops of phenolphthalein, and titrating with N/1 sodium hydroxide, shaking vigorously until the solution remained faintly but permanently pink. It was then diluted to 10 per cent. strength with distilled water, and from this, dilutions of 1 in 20, 1 in 40, etc., to 1 in 640 were prepared with distilled water as they were required for use.

PREPARATION OF PNEUMOCOCCUS CULTURES.

The stock strains of pneumococci were kept in broth containing 10 per cent. defibrinated horse-blood in the ice-chest, and were subcultured into hormone broth tubes, which were then used to inoculate flasks of the same medium. These cultures were allowed to grow from 6 to 16 hours before use, and before the tests were put up each culture was plated on blood-agar and the reaction was adjusted to pH 7·6. This adjustment of the reaction was necessary in order to avoid precipitation of the bile-salts by the acidity of the medium.

METHOD OF TESTING.

0·1 c.c. of each bile-salt dilution was added to 0·9 c.c. of culture, and the mixture was allowed to stand for one hour at room temperature before the final reading was made, although clearing was usually apparent in those tubes containing the higher concentrations of bile-salts within a few minutes.

TABLE I.

Bile-salts. Sodium salts of—	Final dilutions.* 1 in 100.	1 in 200.	1 in 400.	1 in 800.	1 in 1600.	1 in 3200.	1 in 6400.
A. *Uncombined bile-acids.*							
Cholic acid	+	+	+	∓	−	−	−
Dehydrocholic acid	−	−	−	−	−	−	−
Dehydrodeoxycholic acid	−	−	−	−	−	−	−
Apocholic acid	+	+	+	+	+	−	−
Deoxycholic acid	+	+	+	+	+	+	−
B. *Choleic acids.*							
Acetic choleic acid	+	+	+	+	+	∓	−
Propionic choleic acid	+	+	+	+	+	∓	−
Palmitic choleic acid	p	p	p	p+	+	±	−
Stearic choleic acid	p	p	p	p+	+	±	−
Oleic choleic acid	+	+	+	+	+	+	−
Linoleic choleic acid	+	+	+	+	+	+	−
Tributyrin choleic acid	+	+	+	+	+	∓	−
Triolein choleic acid	p	p	p+	+	+	±	−
Acetone choleic acid	+	+	+	+	+	±	−
Ether choleic acid	+	+	+	+	+	±	−
Benzene choleic acid	+	+	+	+	+	+	−
Xylol choleic acid	+	+	+	+	+	∓	−
Benzaldehyde choleic acid	+	+	+	+	+	∓	−
Cholesterol choleic acid	+	+	+	+	+	−	−
Camphor choleic acid	+	+	+	+	+	±	−

+ = complete clearing; ± and ∓ = partial clearing; − = no clearing; p = precipitate.

TESTS WITH SMOOTH STRAINS OF PNEUMOCOCCI.

Table I shows the results of the tests of the 20 bile-salt preparations on a normal smooth pneumococcal strain (No. 14) Group IV. It is not necessary to give tables of the results obtained with the other smooth strains, as they were similar to those obtained for Strain 14, and although the tests were performed on different days, and the results therefore could not be considered strictly comparable, they were remarkably constant.

It will be seen that the salts of deoxycholic acid and the choleic acids were the most active in producing lysis, whilst sodium cholate was much less efficient, a fact which has been noted by others (Mair, 1930). Sodium apocholate was slightly less efficient than sodium deoxycholate, whilst sodium dehydrocholate and sodium dehydrodeoxycholate were inactive, and were always so even when tested in concentrations of 1 in 20. The lytic action of the deoxycholic acid was not apparently affected by the various substances with which it was combined to form choleic acids. With salts of palmitic-choleic, stearic-choleic

* The dilutions shown in all tables represent the concentrations of the original bile-acids and not of the salts formed on neutralization with sodium hydroxide.

and triolein-choleic acids the precipitation indicated in the table always occurred in the same tubes with this and all other strains of pneumococci. The precipitate appeared almost immediately on addition of the bile-salt, but did not prevent the solution of the pneumococci, as examination of the centrifuged deposits from these tubes revealed at most only a few degenerate cocci, while deposits from control tubes with no bile-salt added showed numerous organisms. The precipitation was a zone phenomenon which seemed to depend upon the dilution of these preparations, as it did not occur in dilutions below 1 in 100, or above 1 in 800, and also appeared when dilutions of the salt were made in distilled water or hormone broth, in the absence of pneumococci. The precipitate was not affected by changes in pH within the limits used in the testing of cultures, and was possibly due to contamination with some impurity. It is hoped to prepare further samples of these bile-acids, using specially purified fatty acids.

TESTS WITH ROUGH PNEUMOCOCCUS STRAINS.

Five of the six rough strains tested gave results similar to those obtained with smooth strains, as shown in Table I, but one strain, a rough Type II, was resistant to the action of bile-acids except in very high concentrations, 1 in 20 of sodium deoxycholate or of the choleic acid salts. This particular strain was a stock strain obtained from Dr. Griffith some time previously, but was similar to the other rough strains in its morphology, cultural characters on solid and liquid media, its non-specific agglutination with type sera, and its low degree of virulence for the mouse. Cultures in serum broth of this bile-insoluble rough strain did not show normal autolysis similar to that which occurred with the bile-soluble strains under the same cultural conditions; nor did lysis take place when an autolysate from a lysed culture of a normal strain was added, together with sodium deoxycholate solution, to cultures of this rough strain.

With regard to the bile solubility of rough pneumococcal strains, there would appear to be some variation according to the methods by which the variant strain has been derived. Reimann (1925) has noted that cultures derived from rough colonies obtained after repeated growth in plain broth or broth containing bile or immune serum were less soluble than the original smooth cultures, and that the pneumococci could become so acclimatized to the presence of bile in culture media that they would finally grow in a medium containing 75 per cent. of ox bile. On the other hand, he states (Reimann, 1927) that rough strains obtained after prolonged growth of cultures within the bodies of animals were bile-soluble like normal strains. Griffith (1923) found that rough strains obtained from cultures in homologous immune serum had not become resistant to the action of bile. Morgenroth, Schnitzer and Berger (1925) found that pneumococci became relatively insoluble in bile after exposure to certain drugs, and Atkin (1926) observed that the pneumococci from the papillæ which had resisted normal autolysis in serum-agar cultures were insoluble, but on subculture gave rise to organisms which showed normal autolysis and were lysed by sodium deoxycholate in the usual way. It would seem from the results recorded above and the observations of others, that while rough pneumococci obtained by growth in homologous immune serum

are usually bile-soluble to the same degree as the original strain, it is possible by this and other procedures to produce variants which are more resistant to the action of bile-salts. While, therefore, variation in the bile solubility of degraded pneumococcus cultures would appear in some instances to be associated with loss of virulence and capsule formation, it does not happen that loss of these properties is always associated with alteration in the susceptibility of the organisms to lysis by bile-salts.

TABLE II.—*Effect of Temperature on Lysis. Strain* 14, *Group IV. Dilution of* 1 *in* 1600 *of Sodium Deoxycholate.*

Temperature.	Time required for complete lysis.
0° C.	135 minutes.
10° C.	90 ,,
21° C.	60 ,,
37° C.	45 ,,
45° C.	180 ,,

EFFECT OF TEMPERATURE ON LYSIS.

Owing to the difficulty of determining exactly when complete lysis has taken place, it is not claimed that the results shown in Table II on the effect of temperature on lysis show a high degree of accuracy, but it will be seen that, as Goebel and Avery (1929) state, lysis takes place at 0° C., though more slowly than at higher temperatures. It was also observed in experiments made with various dilutions of the same bile-salt at 0° C. that at this temperature lysis is brought about more rapidly by the higher concentrations of bile-salt. It will also be seen that up to 37° C. the rate at which lysis takes place increases with rise of temperature, and decreases on raising the temperature to 45° C. This is in agreement with the findings on the relation of temperature to normal autolysis.

SURFACE TENSION IN RELATION TO LYSIS.

One of the principal properties of the bile-salts is their effect upon the surface tension of their solutions. In the investigation of this factor other substances which lower surface tension were tested with cultures of Strain 14 for their lytic action. Sapotoxin caused complete lysis in dilutions from 1 in 20 to 1 in 800. Sodium oleate did not produce lysis within an hour in a dilution of 1 in 20. It has already been mentioned that sodium dehydrocholate and sodium dehydrodeoxycholate were inactive in this dilution.

The result with sodium oleate is in agreement with Lamar (1911), who found that, in a dilution of 1 in 1000, it did not produce complete lysis of pneumococci in one hour. Falk and Yang (1926), however, found that suspensions of washed pneumococci were lysed by solutions of sodium oleate of 1·25 per cent. strength and higher. The same authors found that sapotoxin in concentrations of 1·25 per cent. and higher did not lyse pneumococci—a result

which our tests do not confirm. It is possible that the preparation of sapotoxin used by us was more active than the saponin used by Falk and Yang.

TABLE III.

Salt.	Limiting dilution for lysis.	Surface tension of solutions of approximately similar strength.	
		Dilution.	Surface tension.
Sodium deoxycholate	1 in 3200	1 in 2551[1]	63·18 dynes per cm.
		1 in 3826	66·36 ,, ,,
Sodium cholate	1 in 400	1 in 245[1]	56·64 ,, ,,
		1 in 612	60·11 ,, ,,
,, apocholate	1 in 1600	1 in 1282[1]	58·38 ,, ,,
		1 in 2564	63·62 ,, ,,
,, dehydrocholate	Inactive	1 in 25[1]	55·22 ,, ,,
	1 in 20	1 in 62	58·75 ,, ,,
,, oleate	Inactive		
	1 in 20	1 in 1000[2]	22·55 ,, ,,

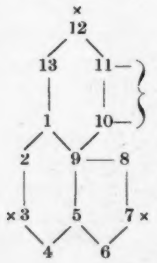
[1] From E. Gillert (1926).—[2] From Walker (1921).

Table III gives, for certain substances which have been tested, the limiting dilution which lysed pneumococci, and the surface tension of solutions of as similar strength as have been measured.

Allowing for the difficulty in observing the exact dilution which will just produce complete lysis, it will be seen that there is a certain similarity in the surface tension of such solutions of sodium deoxycholate, sodium cholate, and sodium apocholate, but on the other hand these solutions have a higher surface tension than solutions of sodium dehydrocholate, which failed to produce lysis in a concentration of 1 in 20. Further, a 1 in 1000 solution of sodium oleate has an altogether lower surface tension than any solution of the bile-acids we have tested, yet in our experiments sodium oleate failed to lyse pneumococci. It would thus appear that although the surface activity of bile-salts may play a part in lysis of pneumococci, a lowering of surface tension alone will not explain the phenomenon.

LYSIS AND THE CHEMICAL STRUCTURE OF THE BILE-ACIDS.

The lytic activity of the bile-acids appears to be related to the number and position of the hydroxyl groups. Of the five uncombined bile-acids tested, four are saturated and have the common carbon framework :



whilst that of the fifth, apocholic acid, differs only in having a double bond at an as yet unascertained position.

Deoxycholic acid ($C_{24}H_{40}O_4$) and apocholic acid ($C_{24}H_{38}O_4$) are dihydroxy-acids, in both of which the hydroxyl groups are situated at positions 3 and 7. Cholic acid ($C_{24}H_{40}O_5$), a trihydroxy-acid, has hydroxyl groups at positions 3, 7 and 12. Dehydrodeoxycholic acid ($C_{24}H_{36}O_4$) is the diketo-acid derived from deoxycholic acid, and dehydrocholic acid ($C_{24}H_{34}O_5$) is the triketo-acid derived from cholic acid.

From Tables I and III it will be seen that the salts of dehydrocholic acid and dehydrodeoxycholic acid, which have no hydroxyl groups, are inactive, whilst those of deoxycholic acid and apocholic acid, which possess hydroxyl groups at positions 3 and 7 only, are more active than that of cholic acid, which possesses a third hydroxyl group at position 12.

A remarkable property of some, though not all, bile-acids is their ability to form addition compounds of the choleic acid type. The two most active uncombined bile-acids we have tested, deoxycholic acid and apocholic acid, both form stable addition compounds with various organic substances (Rheinboldt, 1928), whilst the much less active cholic acid forms only very weak addition compounds (Rheinboldt, 1929), and the two completely inactive bile-acids, dehydrocholic acid and dehydrodeoxycholic acid, do not form these compounds.

Sapotoxin is also able to form addition compounds with such substances as cholesterol and lecithin.

The data available are not sufficient for definite conclusions, but it seems possible that this power of forming addition compounds may have some bearing on the mechanism of bile solubility of pneumococcus.

DISCUSSION.

It has been pointed out by Mair (1930) and others that the conditions under which lysis by bile-salts and normal autolysis take place are similar. Alteration in temperature has a corresponding effect on the two processes, and it is interesting to note that the rough strain which did not undergo spontaneous autolysis in the present experiments was resistant to autolysin from another pneumococcal strain, and also resisted the action of bile-salts. Further, it is known that although cultures of pneumococci which have been killed by heat and in which the normal autolytic enzyme has been destroyed resist the lytic action of bile-salts, when a lysed culture is added, solution of the heat-killed organisms occurs in the usual manner and the addition of bile hastens the reaction. It has been observed that autolysis of pneumococci can be hastened by the addition of chloroform and similar lipoid solvents, and it has been suggested by Lamar (1911) that the action of sodium oleate in rendering the pneumococcus more susceptible to autolysis is due to the damage of the cell membrane. Lord and Nye (1922) believe that the action of the bile-salts is of a similar nature. It is interesting to note that autolysis of meningococci, which normally takes place slowly as a result of the action of an intracellular enzyme, can be hastened by the addition of toluol or substances of a similar nature (Murray, 1929).

On the other hand, the fact that lysis by bile-salts and normal autolysis occurs best under similar conditions, might merely be an indication that under these conditions the pneumococcus is particularly susceptible to disruption by enzyme action or by chemical agents independently of the autolytic enzyme. Goebel and Avery (1929) have shown that solution of pneumococci by bile-salts is not always accompanied by the same proteolysis which takes place during normal autolysis; yet as lysis of pneumococci by bile-salts does not apparently take place in the absence of autolysin, it seems possible that bile-salts accelerate the normal autolytic process due to enzyme action, although, as Goebel and Avery have found, the final proteolytic changes associated with normal autolysis may be inhibited by the presence of the bile-salts in high concentrations.

It has been shown above that the presence and position of hydroxyl groups in the bile-acids and the power to form addition compounds with organic substances is associated with the ability to produce rapid lysis of pneumococci under suitable conditions. The observations of others discussed above and the experiments made in connection with this work suggest that the bile-acids combine with the pneumococcal cell and so alter it that lysis is readily produced by the autolytic enzyme of the cell, although the other possibility, that the bile-salts act independently of the autolytic enzyme as indicated by Goebel and Avery, has not been disproved.

SUMMARY.

1. The preparation of various derivatives of bile-acids has been described, and tests of the lytic action of these on strains of pneumococci from various sources and of different serological types have been carried out.

2. All normal strains of pneumococci tested, irrespective of type, were equally susceptible to the lytic action of the various bile-salts. Of six rough strains tested, five were equally soluble, while the sixth was almost completely resistant.

3. Comparative tests with various bile-salts and sodium oleate indicate that there is no parallelism between lytic activity and the surface tension of solutions.

4. Of the substances tested, saponin and the sodium salts of all the bile-acids except dehydrocholic acid and dehydrodeoxycholic acid brought about lysis. The fact that the sodium salts of the various choleic acids were similar in their activity to sodium deoxycholate indicates that the former owed their activity to the deoxycholic acid fraction.

5. There would seem to be some correlation between the chemical structure and power to form addition compounds and the lytic activity of the various bile-salts tested.

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THE FRACTIONATION OF ANTI-VACCINIA SERUM.

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As the result of recent work there is now a considerable body of evidence to show that the immunity reactions occasioned by ultra-microscopic viruses are essentially similar to those produced by bacteria. In the case of bacteriotropic sera the properties which are responsible for agglutination and phagocytosis are, it is well known, to be found in the globulin fraction of the sera. Observations of a similar nature in regard to antiviral sera are at present lacking.

In 1899, however, Béclère, Chambon and Ménard published the results of their investigations on the capacity of the serum of an animal actively immunized against vaccinia to inactivate that virus *in vitro*. They showed that the substance in the serum upon which this antiviral property depends is attached to the globulin fraction.

As the question is one of both practical and theoretical interest, there are here described investigations which were undertaken to determine in which fraction of a vaccinia immune serum the following antiviral properties are contained: (i) the protective action of immune serum *in vivo*, (ii) the flocculating action of immune serum on vaccinia virus *in vitro*, (iii) the effect of immune serum on the phagocytosis of vaccinia virus *in vitro*.

TECHNIQUE.

An antivaccinial serum was prepared in four rabbits, using the method recommended by Gordon (1925), and more recently by Burgess, Craigie and Tulloch (1929). Vaccinia virus which had been repeatedly passaged on the

skin of rabbits was employed, the rabbits being injected either intravenously or intraperitoneally at short intervals for a period of two months. After preliminary observations to test the flocculating power of the sera the animals were bled out under ether anæsthesia. The sera were then pooled, the resultant volume being divided into two equal parts. One of these was stored aseptically in the ice-chest, the other was fractionated with ammonium sulphate. Before carrying out the fractionation the serum was first diluted with an equal volume of 0·85 per cent. sodium chloride solution. To one volume of the serum thus diluted, one volume of a 66 per cent. saturated solution of ammonium sulphate was added. The mixture after shaking thoroughly and allowing to stand for 15 minutes at room temperature was filtered in the ice-box. This first precipitate consists of the " euglobulin " fraction.

To the clear filtrate enough ammonium sulphate was then added to bring the concentration up to 50 per cent. saturation ; the mixture was again filtered in the ice-box, this fraction constituting the " pseudoglobulin." The clear filtrate was now saturated with ammonium sulphate and the precipitate obtained by filtration. This precipitate constituted the albumin fraction.

After drying, the precipitates were dissolved in distilled water and dialysed in parchment thimbles for a week against large quantities of distilled water. After the completion of dialysis the three fractions were each reduced to a volume equal to that of the original serum, using an electric fan to increase the rapidity of evaporation. Enough sodium chloride was added to make 0·85 per cent. solutions.

The action of the three fractions and of the whole serum was then compared in the following experiments :

THE PROTECTIVE ACTION OF THE PROTEIN FRACTIONS.

To test the protective action of the protein fractions of the immune serum a dilution of vaccinia virus 1 in 10 in physiological saline was mixed with an equal volume of increasing dilutions of whole serum and of the protein fractions in physiological saline. The mixtures were allowed to stand for an hour in the incubator at 37° C., then 0·1 c.c. of each mixture was inoculated intradermally into the skin of the abdomen of a rabbit. The results are shown in Table I :

TABLE I.

Dilution.	Whole serum.	Euglobulin fraction.	Pseudoglobulin fraction.	Albumin fraction.
1 in 2	—	—	—	(+)
1 in 5	—	—	—	+
1 in 10	—	—	(+)	+
1 in 20	—	—	+	+
1 in 30	—	+	+	+
1 in 40	—	+	+	+
1 in 50	+	+	+	+

— = No reaction. (+) = Very slight reaction. + = Typical vaccinia reaction.

While whole serum protected up to a dilution of 1 in 40, euglobulin was active up to a dilution of 1 in 20, pseudoglobulin up to 1 in 5, with a doubtful reaction in a dilution of 1 in 10. The albumin fraction was only doubtfully protective in a dilution of 1 in 2.

THE FLOCCULATING ACTION OF THE PROTEIN FRACTIONS.

To test the flocculating action of the various protein fractions, dilutions were mixed with a suspension of vaccinia virus 1 in 10 and were placed in the water-bath at 55° C. for 24 hours, the method being essentially that employed by Burgess, Craigie and Tulloch (1929). The results are seen in Table II :

TABLE II.

Dilution.	Whole serum.	Euglobulin fraction.	Pseudoglobulin fraction.	Albumin fraction.
1 in 5	+	+	+	—
1 in 10	+	+	+	—
1 in 20	+	+	—	—
1 in 25	+	—	—	—
1 in 30	+	—	—	—
1 in 40	—	—	—	—

+ = Flocculation. — = No flocculation.

As in the case of the experiment on the protective action of the serum fractions, euglobulin was more efficient than pseudoglobulin while the albumin fraction gave no flocculation.

THE EFFECT OF THE SERUM FRACTIONS ON PHAGOCYTOSIS.

Douglas and Smith (1930) have brought forward evidence to suggest that in the presence of vaccinia immune serum the leucocytes of the rabbit have an increased capacity for fixing and destroying vaccinia virus.

An attempt was made to determine with which fraction of the serum proteins this enhancing effect was associated. A normal rabbit was therefore bled, heparin (2 gm. per litre) being used as an anti-coagulant. The blood was centrifuged, the plasma pipetted off, and the leucocyte layer removed. The leucocytes were washed in saline and again centrifuged, the supernatant fluid being pipetted off. One volume of leucocytes was then mixed with an equal volume of the following : (i) normal rabbit serum ; (ii) immune anti-vaccinia rabbit serum ; (iii) euglobulin fraction of immune antivaccinia serum ; (iv) pseudo-globulin fraction of immune antivaccinia serum ; (v) albumin fraction of immune antivaccinia serum.

The mixtures, which were thoroughly shaken at intervals, were kept for an hour at 37° C. ; they were then centrifuged and the supernatant fluid pipetted off. The five samples of treated leucocytes were then added to an equal volume of vaccinia virus diluted 1 in 10 in physiological saline. The mixtures were hermetically sealed in small glass tubes and were attached to

a wooden paddle-wheel, which was slowly revolved in a water-bath at 37° C., thus ensuring an adequate mixing of the cells with the virus suspension. At the end of 15 minutes the contents of the tubes were removed and centrifuged, dilutions of the supernatant fluids and of the cellular deposits being inoculated intradermally into rabbits.

The results are shown in Table III :

TABLE III.

Dilutions.	Vaccinia virus and leucocytes treated with normal serum.		Vaccinia virus and leucocytes treated with vaccinia immune serum.		Vaccinia virus and leucocytes treated with euglobulin fraction of immune serum.		Vaccinia virus and leucocytes treated with pseudoglobulin fraction of immune serum.		Vaccinia virus and leucocytes treated with albumin fraction of immune serum.	
	Supernatant fluid.	Cells.	Supernatant fluid.	Cells.	Supernatant fluid.	Cells.	Supernatant fluid	Cells.	Supernatant fluids.	Cells.
1 in 2	+	+++	−	+	−	+	−	+	+	++
1 in 4	−	++	−	−	−	+	−	+	−	++
1 in 10	−	+	−	−	−	−	−	+	−	+
1 in 20	−	+	−	−	−	−	−	−	−	+

These experiments confirm the results obtained by Douglas and Smith (1930) in regard to the action of an immune antivaccinia serum on the phagocytosis of vaccinia virus. They also show that while this action is associated with the euglobulin and pseudoglobulin fractions of the immune serum, it is not found in relation to the albumin fraction.

DISCUSSION.

The observations here recorded confirm those originally made by Béclère, Chambon and Ménard (1899) as to the relation between the globulin fraction of the serum of a rabbit immunized against vaccinia and the power of the serum to inactivate vaccinia virus ; the antibody also resists dialysis. In addition the experiments show that the flocculating antibody and the power to increase the phagocytosis of vaccinia virus are also associated with the globulin fraction of the serum. In this particular, therefore, vaccinia virus is similar to bacteria in that the immune body in bacterial diseases is usually associated with the globulin fraction of the serum.

In vaccinia the greater part of the immune body appears to be associated with the euglobulin fraction ; part, however, with the pseudoglobulin fraction. There are two possible explanations of this fact. Many observers, such as Chick (1914) and Sørensen (1925), believe that it is impossible to obtain complete separation of euglobulin and pseudoglobulin. Sørensen, for instance, is of opinion that euglobulin and pseudoglobulin are associated as a labile compound, E*p*P*q*, with the result that neither fractionation nor dialysis, nor a combination of the two methods, is capable of completely separating the two globulins.

The second explanation is that the immune body is not itself a protein, but an enzyme which is adsorbed in varying proportions by the proteins which together constitute the globulin fraction of the serum.

CONCLUSIONS.

In antivaccinia immune serum prepared in the rabbit, the immune body responsible for the protective action of the serum, for the flocculation of vaccinia virus and for the action stimulating the phagocytosis of vaccinia virus is associated with the globulin fraction of the serum.

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THE EFFECT OF SUBCUTANEOUS INJECTIONS OF TRYPSIN ON THE BLOOD-SUGAR AND ON INSULIN ACTION.

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The pathologic process underlying the comparative failure of insulin to lower the blood-sugar in diabetic patients who are suffering from an acute infection remains unexplained. Whether it be due to a direct neutralization of insulin in the blood-stream, to an inability on the part of the organism to store carbohydrate, or to an increased tendency to glycogenolysis (Epstein and Rosenthal, 1925; Tisdall, Drake and Brown, 1926; Lawrence and Buckley, 1927; Sweeney and Lackey, 1928) is not discussed in this paper. But an attempt has been made to find what factor in the inflammatory or toxic process is responsible for the relative failure of insulin.

The proteolytic ferments in inflammatory exudates have been suggested as responsible for the weakened action of insulin in these cases. Rosenthal and Behrendt (1926) have shown that insulin which has been left in contact with pus becomes physiologically inactive. Similarly no fall of blood-sugar occurs when insulin and trypsin, or insulin and pepsin, are mixed at a suitable hydrogen ion concentration, and subsequently injected into rabbits (Epstein

* Receiving a part-time grant from the Medical Research Council.

and Rosenthal, 1924; Harteneck and Schuler, 1927). In the following experiments an artificial excess of trypsin in the organism has been produced in order to discover whether an increase of proteolytic ferment in the body will inhibit the action of insulin.

The immediate hyperglycæmic effect of intravenous injections of trypsin into rabbits has been described by Epstein and Rosenthal (1925). The same transitory rise of blood-sugar has been found in many of our animals when trypsin was injected subcutaneously. In addition it has been found that for a variable number of days following the injection of a single dose of trypsin, insulin either has no effect on the blood-sugar or causes only a small depression; the normal response, however, gradually returns.

The immediate transitory hyperglycæmic action of a single subcutaneous dose of trypsin, and the extent of impairment of the response to insulin on subsequent days, appear to bear a relation to the degree of activity of the ferment. Trypsin which has been partially inactivated by heat has less hyperglycæmic effect and less power to inhibit insulin action on subsequent days. It has been found that a rabbit no longer responds in the same way to injections of trypsin when once it has received an injection of partially inactivated ferment. To avoid confusion, experiments where the above conditions were present have been omitted.

METHODS.

Grey or brown male rabbits, weighing about 2 kg., were used as the experimental animals. Their food, consisting of a daily ration of oats and cabbage, was taken out of the cage about 17 hours before any blood-sugar test, if not already eaten.

A preliminary blood-sugar curve, following the subcutaneous injection of one unit of insulin, was made as a control to later experiments. On a subsequent day a subcutaneous injection of trypsin was given. The effect on the blood-sugar during the following 2 or 3 hours was observed, and also the effect of one unit of insulin on the blood-sugar level on succeeding days, until the response had returned to normal. A blood sample was obtained before the injection, whether of trypsin or of insulin, and at half-hourly intervals after for 2 hours or more. Blood was obtained by heart puncture and the sugar content estimated in duplicate by Maclean's method. The rectal temperature was taken at the time of each blood-sugar test.

Fairchild's sterile glycerinated trypsin was used in all cases: 1/20 or 1/10 c.c. diluted to 1 c.c. was injected into the subcutaneous tissue of the flank. There were, as a rule, no obvious constitutional effects following the injection. A few rabbits, however, showed immediate symptoms of discomfort. The respiration became panting, the animal lay flat and limp, and blood was occasionally found on the thermometer after taking the rectal temperature. These effects, when present, cleared up within an hour. They were associated with larger rises of blood-sugar than were found in rabbits not so affected, and were more liable to occur after larger doses of trypsin.

The insulin used belonged to brands supplied by Boots' or Burroughs Wellcome. Four units were diluted to five times their volume, and one unit

TABLE I.—*Blood-sugar (mgm. per 100 c.c.) and Temperature (Fahrenheit) after Injections of Trypsin and Insulin.*

	Rabbit.	Date.	Injection.	Before.		½ hour.		1 hour.		1½ hours.		2 hours.		3 hours.		Maximum change of blood-sugar.
A	31	16.5.27	1 unit insulin . .	109	101·7°	93	101·4°	56	101·9°	54	101·3°	81	101·6°	..	..	− 55
		2.2.28	1/10 c.c. trypsin . .	118	102·1°	119	101·5°	114	101·3°	160	101·2°	197	102·0°	..	..	+ 79
		15.2.28	1/5 c.c. ,, . .	138	101·2°	354	99·2°	340	99·2°	446	98·2°	380	98·6°	283	101·2°	+308
		20.2.28	1/10 c.c. ,, . .	134	101·1°	248	100·0°	265	99·8°	..	..	221	101·0°	..	..	+131
B	38	21.6.28	1 unit insulin . . .	110	102·2°	97	102·0°	62	101·8°	58	101·8°	31	101·9°	..	..	− 79
		25.6.28	1/20 c.c. trypsin . .	167	102·5°	175	100·8°	204	100·2°	189	100·5°	164	101·0°	..	..	+ 37
		26.6.28	1/20 c.c. ,, . .	122	101·8°	124	101·8°	135	102·0°	124	102·0°	112	102·0°	120	102·4°	+ 13
			1 unit insulin													
		27.6.28	1 ,, ,, . . .	109	103·1°	106	102·4°	90	102·4°	98	102·4°	95	102·4°	121	102·6°	− 19
		28.6.28	1 ,, ,, . . .	108	103·3°	95	103·0°	58	103·2°	71	103·0°	58	103·0°	83	103·0°	− 50
C	38	21.6.28	1 unit insulin . . .	110	102·2°	97	102·0°	62	101·8°	58	101·8°	31	101·9°	..	..	− 79
		29.6.28	1/20 c.c. trypsin heated to 56° C. for 10 mins.	119	103·2°	127	103·1°	127	102·8°	127	103·0°	121	103·0°	122	103·5°	+ 8
		3.7.28	1/20 c.c. heated trypsin .	133	103·2°	113	102·8°	131	103·0°	117	103·1°	107	103·1°	112	103·2°	− 26
			1 unit insulin													
		4.7.28	1 ,, ,, . . .	91	102·8°	100	103·2°	97	102·9°	104	103·0°	..	102·6°	103	102·8°	+ 13
		5.7.28	1 ,, ,, . . .	127	103·3°	..	103·1°	57	102·9°	44	102·9°	53	102·4°	85	102·4°	− 83
D	47	12.4.29	1 unit insulin . . .	136	102·0°	78	101·8°	69	101·1°	50	100·9°	49	100·8°	..	..	− 87
		15.4.29	1/10 c.c. trypsin heated to 56° C. for 48 hours	115	102·5°	117	102·2°	119	101·9°	125	101·7°	..	..	..	..	+ 10
		16.4.29	1 unit insulin . . .	126	102·9°	79	101·9°	83	100·9°	66	100·5°	64	101·3°	..	..	− 62
		17.4.29	1/10 c.c. heated trypsin .	127	102·6°	116	99·9°	62	100·9°	67	101·8°	60	102·2°	..	..	− 67
			1 unit insulin													

only was injected. This was given subcutaneously into the opposite flank to that used for trypsin injection.

In a second series of experiments the trypsin was partially inactivated by heating to 56° C. for 10 to 30 minutes; and in a third series, trypsin more completely inactivated by heating to 56° C. for 44 to 48 hours was injected.* The second and third series of experiments were undertaken to show that the characteristic effects found with trypsin were related to the activity of the ferment, and not to any other factor involved.

Table I shows (A) the rise of blood-sugar which occurs after subcutaneous injections of trypsin; (B) the delayed inhibitory effect on insulin action on days following a subcutaneous injection of trypsin, and the failure of insulin to affect the blood-sugar when trypsin is injected at the same time at a different part of the body; (C) the less marked hyperglycæmic and insulin-inhibitory effects of a weaker ferment; and (D) the absence of an inhibitory effect on insulin action after the injection of trypsin which has been still more completely inactivated by heat.

TABLE II.—*Summary of all Experiments.*

		Temporary rise of B.S. after injection of trypsin.	Inhibitory effect on the action of insulin when the latter is injected simultaneously with trypsin.	Delayed inhibitory effect on the action of insulin for one or more days after the injection of trypsin.
Active trypsin (Table I, A and B	Present	10	3	3
	Absent	2	1	1
Trypsin heated to 56° C. for 10 to 30 min. (Table I, C)	Present	1	3	2
	Absent	2	1	1
Trypsin heated to 56° C. for 44 to 48 hrs. (Table I, D)	Present	0	0	1
	Absent	3	2	3

As is shown in Table II, similar results were obtained in the majority of our experiments.

CONCLUSIONS.

The small number of experiments at present carried out allow only tentative conclusions to be drawn. They are as follows:

1. The subcutaneous injection of a single dose of 1/10 c.c. Fairchild's sterile glycerinated trypsin into a rabbit is liable to be followed by a transitory

* The proteolytic activity of the ferment was judged by its ability to digest a gelatin film.

rise in blood-sugar. This effect on the blood-sugar is related to the degree of activity of the ferment.

2. The rise of blood-sugar described masks the effect of insulin given subcutaneously at the same time at a different part of the body. When the trypsin is sufficiently inactivated by heat, insulin lowers the blood-sugar in the normal way.

3. The subcutaneous injection of a single dose of sufficiently potent trypsin is followed by a period of days in which insulin fails to lower the blood-sugar, either completely or partially. Normal insulin action gradually returns.

Though there is no definite proof in these results that proteolytic ferments, such as trypsin, play a part in the state of lessened response to insulin found in septic and toxæmic conditions, it is pointed out that an excess of trypsin in the organism has this same effect on insulin action.

This series of experiments forms part of a programme outlined by Dr. R. D. Lawrence for the investigation of variability in the response to insulin, with special reference to inflammatory and toxæmic conditions. We are indebted to him for help and advice.

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SIMULTANEOUS INFECTION OF THE MONKEY WITH THE VIRUSES OF POLIOMYELITIS AND VACCINIA.

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DURING recent years it has been suggested that infection with one virus, *e. g.* that of vaccinia, might perhaps awaken into activity a latent infection with a hitherto unrecognized virus or, alternatively, modify the character of the lesions produced by a previously known virus. The following investigation with the organisms of vaccinia and poliomyelitis was designed to obtain information on the second point.

When considering the ætiology of post-vaccinal encephalitis, the Vaccination Committee (1928) discussed the possibility that the poliomyelitis virus, activated by or exercising its action during the course of vaccination, might

produce lesions of unusual character, viz. widespread perivascular demyelination without the severe nerve-cell changes normally found in poliomyelitis. Paul (1928) described two cases of clinical disseminated encephalo-myelitis and two of Landry's paralysis. In all four the macroscopic and microscopic lesions were comparable, and considered by him to be similar to those of post-vaccinal encephalitis; from one case of Landry's paralysis the poliomyelitis virus was obtained by monkey-passage. The only experimental work on the influence of vaccination on poliomyelitic infection is that of Thomsen (1912), who claimed that, in monkeys, cutaneous vaccination with a mixture of vaccinia and poliomyelitis viruses led to the development of the typical nervous disease; similar treatment with the poliomyelitic virus alone was not followed by infection.

In the case of another neurotropic virus, Levaditi and Nicolau (1923) found that intravenous inoculation of herpes virus into rabbits with "latent spontaneous vaccinia" (rabbits housed in infected cages), caused the appearance of a vaccinial skin eruption with the presence of virus in the testis or ovary; they regarded this (1925) as one expression of a law regulating the behaviour of neurotropic viruses, one of which is latent at the time of infection with the second. Zurukzoglu (1927) made the converse observation that a latent herpes infection might be activated by vaccinia. Of three rabbits receiving herpes virus subcutaneously, three vaccinia intravenously, and three the combined inoculations, only the last group developed herpetic lesions; two of these three animals succumbed to herpetic encephalitis.

EXPERIMENTAL WORK.

The characteristics of the various strains of poliomyelitis virus employed in this investigation have been outlined elsewhere (Fairbrother, 1929); briefly, the "Fl. M." and "Ay" strains are very potent and consistent in their action, while the "A. M." is a weak and inconsistent virus. The neurovaccine was passage-material from that used in our study of vaccinial encephalitis (Hurst and Fairbrother, 1930), and on intradermal titration in rabbits frequently gave titres of 1 in 10,000,000 or higher.

A. *Intracerebral Inoculation into Monkeys of Poliomyelitis Virus Combined with Skin Vaccination.*

M. B5 received intracerebrally 0·5 c.c. 5 per cent. Berkefeld N filtrate of the "A. M." virus and, at the same time, 0·2 c.c. 1 per cent. neurovaccine into the skin of the abdomen. The usual severe skin reaction followed, and the black necrotic area spread progressively, with increasing cachexia and death on 14th day. Histologically the central nervous system was normal.

M. 405 received intradermally 0·2 c.c. 5 per cent. neurovaccine in each of two skin areas and, two days later, 0·5 c.c. 5 per cent. "Fl. M." emulsion into the left cerebral hemisphere. Four days later the vaccinated areas showed an intense reaction and early weakness of the legs was apparent. The paralysis progressed, and the animal was killed when moribund on the 9th day of the experiment. Intradermal titration in rabbits revealed no vaccine virus

in any part of the central nervous system. The histological picture was that of typical poliomyelitis.

Thus no evidence was obtained of the ability of skin vaccination either to activate a weak poliomyelitis virus injected intracerebrally, or to modify the lesions produced by a virulent virus exercising its pathogenic properties during the height of the vaccinial reaction. It will be noted that in the second case no vaccine virus could be detected in the central nervous system.

B. *Combined Intravenous Inoculation of Poliomyelitis and Vaccinia Viruses.*

Clark, Fraser and Amoss (1914) and Flexner and Amoss (1914, *a* and *b*) have shown that intravenous inoculation of any but overwhelming doses of the poliomyelitis virus does not cause the disease unless accompanied by disturbance of the "meningo-choroidal complex"; using a virulent virus the blood may remain infective for at least 72 hours. Table I records experiments

TABLE I.

Animal.	Poliomyelitis virus.	Neurovaccine.	Saline.	Development of nervous disease.
M. 168	..	5 c.c., 0·5% emulsion	..	0
M. 169	10 c.c., 5% Seitz filtrate	..	..	0
M. 170	5 c.c. ,,	5 c.c., 0·5% emulsion	..	0
M. 182	5 c.c., 20% Berkefeld filtrate	..	5 c.c.	0
M. 183	5 c.c. Do.	5 c.c., 5% emulsion	..	0
M. 184	..	5 c.c. ,,	5 c.c.	0
M. 237	5 c.c., 20% emulsion	5 c.c. ,,	..	0
M. 238	5 c.c. ,,	5 c.c. ,,	..	0

made to determine whether simultaneous inoculation with vaccinia may break down the hæmato-encephalitic barrier. On each occasion the poliomyelitis virus ("Fl. M." strain) was tested by intracerebral inoculation in monkeys and the vaccinia virus by intracerebral inoculation and intradermal titration in rabbits and proved to be active.

M. 238 died on the 17th day of sepsis, with no abnormality in the central nervous system. Six weeks later M. 183 succumbed to an intracerebral injection of poliomyelitis virus, showing that no immunity had been acquired.

It will be seen that infection with the virus of vaccinia does not assist the poliomyelitis virus to penetrate the hæmato-encephalitic barrier.

C. *Combined Intracerebral Inoculation of Poliomyelitis and Vaccinia Viruses.*

M. B7 received intracerebrally 0·5 c.c. 5 per cent. "Ay" emulsion and 0·5 c.c. 1 per cent. neurovaccine. It died on the 6th day with the usual clinical and histological picture of cerebral vaccinia. No poliomyelitic lesions were detected in the brain-stem or cord.

M. B8 received 0·5 c.c. 5 per cent. " Ay " emulsion and 0·5 c.c. 0·01 per cent. neurovaccine. It died on the 8th day with the usual clinical and histological picture of cerebral vaccinia. No poliomyelitic lesions were present in the brain-stem or cord.

M. B6 received 0·5 c.c. 5 per cent. " A.M." filtrate and 0·5 c.c. 0·01 per cent. neurovaccine. After a rise of temperature on the 5th and 6th days it recovered and was alive and well five weeks later.

M. 189 received 0·5 c.c. 5 per cent. " Fl. M." emulsion and 0·5 c.c. 1 per cent. neurovaccine. It died on the 5th day, and histologically showed lesions both of poliomyelitis and of vaccinia, the former in the cord and brain-stem, the latter chiefly in the cortex. The changes were quite typical of the respective diseases, neither of which appeared to be modified by the other.

M. 193 received 0·5 c.c. 5 per cent. " Fl. M." emulsion and, two days later, 0·5 c.c. of 1 per cent. neurovaccine. It died on the 7th day with histological appearances similar to those in the preceding case.

These results indicate that animals inoculated intracerebrally with both poliomyelitis and vaccinia virus may, at death, show the lesions of one or both infections, according to the duration of the incubation periods of the respective diseases. Where matters are so arranged that the two conditions evolve together, there is no apparent modification of one by the other. In one experiment the occurrence of cerebral vaccinia did not activate a weak poliomyelitis virus introduced into the brain.

DISCUSSION AND CONCLUSIONS.

It appears that infection with a highly virulent vaccine virus (neurovaccine) has no power to modify the nervous lesions of poliomyelitis ; this is equally true whether the vaccine is injected directly into the brain or inoculated intradermally. If both viruses be administered intravenously, the resulting vaccinial reaction does not suffice to break down the hæmato-encephalitic barrier and permit invasion of the nervous system by the poliomyelitis virus. We have not succeeded in demonstrating an activation effect of vaccinia on a weak poliomyelitis virus.

In applying these negative findings to the problem of post-exanthematous encephalo-myelitis, it must be remembered that much other evidence is opposed to a participation of the poliomyelitis virus in its causation. All investigators, including ourselves,* have failed to obtain this virus from the brains of patients succumbing to post-vaccinal, measles or smallpox encephalo-myelitis ; the description of the lesions in Paul's case of disseminated encephalo-myelitis (clinical Landry's paralysis) is not sufficiently suggestive of the group of diseases under discussion for the unreserved acceptance of his conclusions. In cases of typical poliomyelitis, *occasional* vessels in both the grey and the white matter of the cord may show a well-marked extra-adventitial microglial exudate resembling closely that of post-vaccinal encephalitis ; it is only on staining by the Weigert-Pal method that the complete absence of demyelination clearly indicates the essential difference between the two processes.

* See " Further Report of the Vaccination Committee " (1930), London. H.M. Stationery Office.

Nor has success attended the endeavours of those who have, in this country and in Holland, sought to establish an epidemiological relationship between poliomyelitis and the demyelinating malady. In short, no definite evidence has been brought forward that under any circumstances can the poliomyelitis virus evoke lesions other than those usually associated with its activity.

While it is unsafe to generalize from experience with one neurotropic virus only, the facts recorded in this paper make it unlikely that, if post-vaccinal encephalitis is due to the activation of a latent neurotropic virus by vaccination, any virus hitherto isolated and studied is responsible for the nervous disease.

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THE ABSORPTION OF DIPHTHERIA ANTITOXIN.

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In the clinical use of diphtheria antitoxin, the rate of absorption of antitoxin into the circulation, and the rate of combination of the antitoxin with the toxin present, are the important factors. The first only will be discussed here.

Antitoxin may be present in solutions of different protein concentrations. Walbum (1912) stated that high protein content in the serum retards the absorption of the antibodies by rabbits and, therefore, that concentrated serum is not so efficient as unconcentrated material. This appears to be contradicted by the work of Park, Famulener and Banzhaf (1914), who carried out experiments on goats and men. They concluded that the rate of absorption is the same whether the antitoxin solution injected contained 9·4 per cent. or 18 per cent. of protein. We have endeavoured to determine the influence of such factors as concentration and total protein content on the rate of absorption of antitoxic horse-serum in rabbits and guinea-pigs.

It was necessary to determine the extent to which individual guinea-pigs varied in the rate at which antitoxin is absorbed, and, further, to determine

whether dilution with saline has any effect. Twelve guinea-pigs were injected subcutaneously with 1000 units of diphtheria antitoxin contained in 0·8 c.c of a certain serum, A, and another 12 with the same dose diluted to 5·0 c.c. with saline. At definite intervals after injection, from 3 to 5 c.c. of blood were withdrawn from each guinea-pig, and the antitoxic value of the serum obtained was determined with an accuracy of from 10 to 20 per cent. by the intracutaneous method of testing. The guinea-pigs varied in weight considerably, and therefore, the comparison has been made between the total amount of antitoxin absorbed rather than by the antitoxic value per c.c. of serum. To arrive at the total content, the antitoxic value per c.c. of serum

TABLE I.—*Showing the Total Antitoxin in the Serum of Guinea-pigs at Different Intervals of Time after the Subcutaneous Injection of* 1000 *Units of Diphtheria Antitoxin contained in* 0·8 *c.c. of Undilute Serum—A.*

Guinea-pig.	Weight in grammes.	Total antitoxic content (units).				
		1 hour.	6 hours.	24 hours.	3 days.	7 days.
A	405	2·0	51	182	..	20
B	430	3·2	97	258	..	43
C	470	3·5	47	129	..	16
D	480	6·0	24	168	..	36
E	570	11·4	71	285	228	..
F	630	3·1	63	..	..	..
G	660	2·3	66	231	198	33
H	680	6·8	58	238	..	51
J	750	1·9	30	262	225	37
K	795	7·9	32	159	..	60
L	880	1·8	44	264	220	66
M	880	4·4	35	176	..	66
Average		4·5	52	214	218	43

has been multiplied by the serum volume calculated as one-twentieth of the body-weight.

The results of this experiment are given in full in Tables I and II, which show :

(*a*) That there is a striking difference in the total amount of antitoxin absorbed into the blood-stream by different guinea-pigs injected with the same amount of antitoxin.

(*b*) That this variation is greatest during the first few hours after injection ; thus the amount detected of the total injected is from—

0·17 per cent. to 1·14 per cent. after 1 hour.
2·4 ,, to 9·7 ,, ,, 6 hours.
10·0 ,, to 30·4 ,, ,, 24 hours.

(*c*) That there is no significant difference between the amount of antitoxin absorbed, whether given undiluted or diluted.

These results have been combined in Table III, showing the average content of groups of 8 of the same guinea-pigs divided according to weight. There is no difference in the total amount absorbed in 6 hours by guinea-pigs of different weights, but there is a suggestion that less is absorbed after 24 hours by the lighter animals, of weights varying from 400 to 480 gm., than by the heavier ones, whose weights varied from 570 to 1040 gm.

TABLE II.—*Showing the Total Antitoxin in the Serum of Guinea-pigs at Different Intervals of Time after the Subcutaneous Injection of* 1000 *Units of Diphtheria Antitoxin contained in* 0·8 *c.c. of Serum—A, Diluted to* 5·0 *c.c. with Saline.*

Guinea-pig.	Weight in grammes.	Total antitoxic content (units).				
		1 hour.	6 hours.	24 hours.	3 days.	7 days.
N	400	3·0	28	100	..	24
P	415	3·1	31	145	..	41
Q	420	4·2	57	168	..	21
R	430	6·4	97	204	..	21
S	620	4·6	46	217	139	19
T	790	3·9	39	138	..	31
U	840	1·7	84	210	..	34
V	860	1·7	39	150	129	21
W	870	5·2	87	304	..	43
X	880	1·8	26	176	..	22
Y	1025	2·6	51	153	153	36
Z	1040	2·6	52	260	182	78
Average		3·4	53	185	151	33

TABLE III.—*Showing the Average of the Total Antitoxic Content of Groups of Eight Guinea-pigs of Different Weights after the Subcutaneous Injection of* 1000 *Units of Diphtheria Antitoxin contained in* 0·8 *c.c. of Serum.*

Groups.	Guinea-pigs.	Weight in grammes.	Total antitoxic content (units).				
			1 hour.	6 hours.	24 hours.	3 days.	7 days.
1	A–D, N–R	400–480	3·9	54	169	..	28
2	E–K, S, T	570–795	5·2	51	219	(198)	38
3	L, M, U–Z	840–1040	2·7	52	212	(171)	46

The great variation in individuals at the end of 1 hour is obvious in all the tables, and no conclusions are drawn from any but 6- and 24-hour readings.

It must be pointed out that an accurate comparison cannot be made between the antitoxin content at the end of 24 hours and after 3 days. The apparently smaller content at the end of 3 days is partly due to the withdrawal of a significant amount of antitoxin at the previous bleeding; if we consider guinea-pig "E" in Table I, with a weight of 570 gm., about one-seventh of its total blood volume, and therefore of its antitoxic content, at the time of bleeding has been taken away. At the end of 6 hours, when the total content was

71 units, some 10 units were withdrawn, and therefore the figure of 285 at 24 hours must be increased by some figure slightly less than 10; at 24 hours, 40 units out of 285 were withdrawn, and so the third-day reading would be considerably increased. There is no evidence to show whether individual variation is due to differences in blood content or to differences in the rate of absorption or elimination.

TABLE IV.—*Showing the Relation of Antitoxic Content of Groups of Guinea-pigs Injected with* 1000, 2000 *and* 5000 *Units of Diphtheria Antitoxin contained in* Undilute Serum, *A*.

Groups.	No. of guinea-pigs.	Weight in grammes.	Amount injected.	Total antitoxic content per 1000 units injected.				
				1 hour.	6 hours.	24 hours.	3 days.	7 days.
4	8	570–880	1000 units	5·0	50	231	218	52
5	8	600–1080	2000 ,,	2·7	40	188	188	48
6	4	660–1095	5000 ,,	2·4	31	165	170	53

TABLE V.—*Showing the Average of the Total Antitoxic Content of Groups of Guinea-pigs after the Subcutaneous Injection of* 1000 *Units of Antitoxin contained in* 0·8 *c.c. of Serum A together with* 4·2 *c.c. of Different Non-antitoxic Proteins.*

Groups.	No. of guinea-pigs.	Weight in grammes.	Addition.	Total antitoxic content (units).			
				1 hour.	6 hours.	24 hours.	7 days.
7	8	400–480	*Nil*	3·9	54	169	28
8	16	570–1040	,,	4·0	51	217	42
9	4	400–480	Euglobulin (11 per cent.)	2·2	13	66	37
10	4	790–880	Ditto	4·9	25	114	43
11	4	460–480	Albumin (12 per cent.)	2·0	15	83	21
12	4	650–1020	Ditto	2·6	23	127	50
13	4	450–470	Pseudo-globulin (17 per cent.)	2·3	19	72	29
14	4	730–765	Ditto	1·3	13	94	56

The next experiment was carried out to determine whether the proportional amount of antitoxin absorbed depended on the total amount of antitoxin injected. A further series of guinea-pigs was injected with 2000 and 5000 units of antitoxin contained in 1·6 and 4 c.c. respectively; the same serum, A, was used. The results recorded in Table IV are compared with those for guinea-pigs of corresponding weights given in Table I, and the total antitoxic content is given in terms of 1000 units injected. Table IV shows that a smaller proportional amount of antitoxin is absorbed during the first 24 hours if a larger amount is injected. This result suggests that the rate of absorption following a subcutaneous injection decreases as the total amount of protein

injected increases. In the next experiment, therefore, guinea-pigs were injected subcutaneously with 1000 units of antitoxin contained in 0·8 c.c. of serum, A, together with 4·2 c.c. of a solution of euglobulin, pseudoglobulin or albumin, prepared from normal horse-serum containing no normal antitoxin. The euglobulin solution contained 11 per cent., the pseudoglobulin 17 per cent., and the albumin 12 per cent. of protein. The results are recorded in Table V and show :

(*a*) That the addition of a great excess of protein to the antitoxin injected considerably reduces the amount absorbed 6 or 24 hours after subcutaneous administration.

(*b*) That this reduction is more marked in guinea-pigs of lower weight. The addition of 4·2 c.c. of 11 or 12 per cent. solution of euglobulin or albumin

TABLE VI.—*Showing the Average of the Total Antitoxic Content of Groups of Guinea-pigs after the Subcutaneous Injection of* 2000 *Units of Antitoxin contained in from* 1·33 *to* 6·9 *c.c. of Undilute Antitoxic Serum.*

Groups.	No. of guinea-pigs.	Weight in grammes.	Serum.	Volume.	Total antitoxic content per 1000 units injected.			
					1 hour.	6 hours.	24 hours.	7 days.
15	4	430–500	F	1·33	6·6	71	230	69
16	4	420–490	G	1·43	4·0	38	206	35
5	8	600–1080	A	1·6	2·7	40	188	48
					—	—	—	—
	Average for high-value serum				4·4	50	208	51
17	4	400–470	H	4·5	2·4	36	101	49
18	4	700–730	D	5·0	1·8	31	147	57
19	4	420–500	K	6·9	3·2	29	78	20
					—	—	—	—
	Average for low-value serum				2·5	32	108	42

to 0·8 c.c. of antitoxic horse-serum injected subcutaneously into guinea-pigs of 400–480 gm. reduces the amount of antitoxin absorbed after 6 hours to about 25 per cent. of that absorbed without such addition. In heavier guinea-pigs (650–1050 gm.) the amount absorbed is about 50 per cent. of that without addition. After 24 hours the amount absorbed is 40 to 50 per cent. in the lighter and 50 to 60 per cent. in the heavier guinea-pigs. After the addition of 50 per cent. more protein, 4·2 c.c. of 17 per cent. pseudoglobulin, there is little or no difference in the total amount absorbed by the lighter or heavier guinea-pigs.

These results suggest that the amount of antitoxin absorbed after subcutaneous injection in guinea-pigs will depend on the amount of protein present in relation to the weights of the guinea-pigs, irrespective of the nature of the protein. In the next experiment, groups of four guinea-pigs were injected with 2000 units of antitoxin contained in antitoxic sera of different strengths, ranging from 290 to 1500 units per c.c. These results are given in

Table VI, showing that less antitoxin is absorbed after the subcutaneous injection of low value antitoxin in guinea-pigs than after the injection of the same number of units of high value serum. After 6 hours guinea-pigs have absorbed into the blood-stream 3 per cent. of the total low value serum injected (290–425 units per c.c.), compared with 5 per cent. of the high value (1200–1500 units per c.c.). After 24 hours 10 per cent. of the antitoxin from the low value serum is present, compared with 20 per cent. from the high value.

TABLE VII.—*Showing the Average of the Total Antitoxic Content of Groups of Guinea-pigs after the Subcutaneous Injection of* 100 *and* 400 *Units of High- and Low-Value Antitoxic Sera Diluted to* 5·0 *c.c. with Saline.*

Groups.	No. of guinea-pigs.	Weight in grammes.	Serum.	Units injected.	Volume.	Total antitoxic content per 1000 units injected.	
						6 hours.	24 hours.
20	4	425–475	B	100	0·0225 c.c.	71	283
21	4	435–470	C	100	0·235 ,,	64	215
22	3	400–480	B	400	0·09 ,,	81	282
23	4	430–505	C	400	0·94 ,,	64	278

TABLE VIII.—*Showing the Relationship between the Amount of Protein Injected and the Amount of Antitoxin Absorbed.*

Groups.	No. of animals.	Grammes protein injected per 1000 gm. of body-weight.	Average antitoxic content per 1000 units injected.			
			1 hour.	6 hours.	24 hours.	7 days.
9, 13, 14, 11, 19	20	1·0 to 2·0	2·2	18	79	32
10, 12, 17, 18	16	0·5 to 1·0	2·9	29	122	50
6, 15, 16	12	0·2 to 0·5	4·3	47	200	52
1, 2, 5, 23	28	0·1 to 0·2	3·9	52	213	38
3, 21	12	0·05 to 0·1	2·7	58	213	46
20, 22	7	under 0·05	..	76	282	..

It would appear probable that the conclusions for Table VI are only true if relatively large amounts of antitoxic sera are injected. In the next experiment, therefore, groups of guinea-pigs were injected with 100 and 400 units of antitoxin contained in low-value serum (about 8 per cent. protein and 425 units per c.c.) and in high-value concentrated serum (about 15 per cent. protein and 4500 units per c.c.). The results given in Table VII show that when small amounts of antitoxin are injected subcutaneously into guinea-pigs, the strength of the antitoxin given does not appreciably affect the amount absorbed. The amount of protein injected in the low-value serum was six times that with the high-value, but the difference of amounts absorbed is much less than the difference recorded in Table VI when larger amounts were injected.

In order to consider in closer detail the relation between the amount of protein injected and the amount of antitoxin absorbed in a given time the

results of all groups have been summarized in Table VIII. From this it is seen :

(*a*) That the rate of absorption of antitoxin depends on the amount of protein injected compared with the body-weight of the animal.

(*b*) That the influence, on the rate of absorption, of the amount of protein injected is greatest when the latter is large in relation to the weight of the animal. Thus, the effect of decreasing the initial quantity of protein by one-half, is to increase by over 50 per cent. the amount of antitoxin absorbed. A further increase of over 50 per cent. is obtained by again halving the amount of protein injected, in relation to animal weight. On reducing the latter protein figure to less than one-quarter, however, the absorption of antitoxin increases by less than 50 per cent.

TABLE IX.—*Showing the Total Antitoxic Content of Individual Rabbits after the Subcutaneous Injection of* 2000 *Units Diphtheria Antitoxin contained in Varying Amounts of Antitoxic Serum.*

Rabbit.	Weight in grammes.	Injection.		Total antitoxic content per 1000 units injected.					
		Volume.	Material.	½ hour.	1 hour.	3 hours.	6 hours.	24 hours.	3 days.
1	2000	5·0 c.c.	D. Undilute	..	6·0	..	28	149	..
5	2150	..	..	..	5·4	8	20	134	..
11	2050	..	..	1·0	2·6	..	23	127	230
2	1800	1·6 c.c.	A. Undilute	..	5·4	..	81	326	..
6	2150	..	..	..	4·3	14	64	295	..
12	2250	..	..	0·6	1·9	..	40	255	255
3	1850	0·55 c.c.	E. Undilute	..	2·3	..	65	304	..
7	2200	..	..	..	3·9	13	44	260	..
13	1950	..	..	0·1	2·0	..	48	169	..
4	1850	0·55 c.c.	E. +0·55 c.c. saline	..	5·6	..	75	393	..
8	2400	..	..	..	3·0	12	38	238	..
14	2000	..	..	0·05	0·5	..	53	198	198
9	2150	0·55 c.c.	E. +4·45 c.c. saline	..	2·7	12	37	215	..
15	1800	..	..	0·4	1·8	..	45	180	249
10	2250	0·55 c.c.	E. +4·45 c.c. normal horse serum	..	4·5	20	48	153	..
16	2000	..	..	0·02	0·5	..	35	99	99

Additional evidence of the influence of total protein in relation to the body-weight was given by some experiments on rabbits which are recorded in Table IX.

Ten of the rabbits were given an intravenous injection of 1·0 c.c. of tetanus antitoxin containing 6400 International units (3200 American units) per c.c., at the same time as the subcutaneous injection of diphtheria antitoxin. A comparison between the diphtheria and tetanus antitoxic values of the blood given in Table X shows :

(*a*) That the individual variation in rabbits was not due entirely to variation in blood volume, but that there were differences in the rate of absorption.

(*b*) That the amount of antitoxin absorbed 1 hour after subcutaneous injection varies from 0·2–0·7 per cent. of the amount present after intravenous injection.

(*c*) That 6 hours after injection the amount in the circulation of the antitoxin given subcutaneously varies from 3·3–7·7 per cent. of that given intravenously when the total protein injected is high, and from 5·0–12·7 per cent. with lower amounts of protein.

(*d*) That 24 hours after injection this proportion varies from 33·2–36·0 per cent. for a high protein content and from 42·6–89·7 per cent. for low protein.

Boycott (1912) states that a rabbit of about 2 kilos may be assumed to have 45 c.c. of blood per kilo of body-weight. In this paper we have assumed the serum contained in both guinea-pigs and rabbits to be one-twentieth of the body-weight. In Table XI we have recorded the antitoxic content of the

TABLE X.—*Showing the Relation between the Tetanus and Diphtheria Antitoxic Content of the Blood of Individual Rabbits at Different Intervals of Time after the Intravenous Injection of Tetanus Antitoxin and the Subcutaneous Injection of Diphtheria Antitoxin Calculated according to the Antitoxic Content per* 1000 *Units Injected.*

Rabbit.	Grammes of protein injected subcutaneously per 1000 gm. body-weight.	Percentage amount of antitoxin absorbed from subcutaneous, compared with intravenous injection after—		
		1 hour.	6 hours.	24 hours.
1	0·22	0·5	7·7	36·0
2	0·20	0·7	4·5	34·4
3	0·20	0·6	3·3	33·2
4	0·08	0·6	11·4	76·9
5	0·08	0·5	12·7	79·4
6	0·04	0·2	10·1	69·4
7	0·04	0·4	6·9	57·9
8	0·04	0·63	11·2	89·7
9	0·04	0·3	5·7	53·4
10	0·04	0·3	5·0	42·6

ten rabbits that were given intravenous injections of tetanus antitoxin. This table shows that, on the assumption that the serum volume is one-twentieth of the body-weight, individual rabbits contained from 84–107 per cent. of the amount injected when tested 1 hours afterwards. From 50–70 per cent. was present 6 hours later, and from 37–50 per cent. was present after 24 hours.

Glenny and Hopkins (1922) have shown that after the intravenous injection of antitoxin there occurs a 50 per cent. loss in the first 24 hours, most of which takes place in the first 6–12 hours. The figure of 91 per cent. obtained for the average of the ten rabbits 1 hour after the injection corresponds fairly closely with the theoretical 100 per cent. immediately after injection. Thus the factor of one-twentieth appears to be reasonable as an average figure to take. The majority of individual rabbits vary within 10 per cent. of this figure, and only in one case is the difference greater than this (Rabbit 9).

While considering the practical application of these results, the relative weights of the guinea-pig and man must be considered. When doses of, say,

10,000 units are given to small infants of 10 or 20 kilos weight, the amount of protein per 1000 gm. body-weight is of the order of 0·05 to 0·1 gm. if an average concentrated antitoxin is used. If it is possible to transfer directly our result from the guinea-pig to man with due allowance for weight, then it is obvious that with this scale of dosage in small children or larger doses in adults, the protein content of the material injected can vary within fairly wide limits without appreciably altering the rate of absorption. The difference between concentrated and unconcentrated sera would, however, be appreciable when larger doses are given to small children; concentrated antitoxin of high potency would be absorbed more rapidly than the same number of units of serum of low potency.

TABLE XI.—*Showing the Total Antitoxic Content of Rabbits after the Intravenous Injection of* 1·0 *c.c. Concentrated Tetanus Antitoxic Serum Containing* 6400 *International* (3200 *American*) *Units per c.c.*

Rabbit.	Weight in grammes.	Total antitoxin content per 1000 units injected assuming serum volume to be 1/20th body-weight.		
		1 hour.	6 hours.	24 hours.
1	2000	866	618	433
2	1800	933	707	424
3	1850	879	643	438
4	1850	935	643	438
5	2150	840	605	403
6	2150	840	504	369
7	2200	863	634	449
8	2400	929	669	446
9	2150	1075	739	504
10	2250	957	619	425
	Average	912	638	433

CONCLUSIONS.

1. There is a considerable difference in the total amount of antitoxin absorbed into the blood-stream by different guinea-pigs injected subcutaneously with the same amount of serum. No conclusions can be drawn from experiments upon a limited number of animals.

2. There is no significant difference between the amount of antitoxin absorbed, whether given diluted or undiluted.

3. The rate of absorption of antitoxin depends on the amount of protein injected compared with the body-weight of the animal. The amount of antitoxin absorbed in 6 hours may be reduced, by the addition of a great excess of protein, to about 25 per cent. of that absorbed without such addition.

4. The influence on the rate of absorption of the amount of protein injected is greatest when the latter is large in relation to the weight of the animal.

5. The average concentrated therapeutic serum is absorbed at least as

rapidly as unconcentrated serum containing the same number of units when small doses are injected and more rapidly when large doses are given.

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QUANTITATIVE ASPECTS OF IMMUNITY REACTIONS: THE PRECIPITIN REACTION.

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THE effect of the conditions, under which precipitation takes place, on the amount of precipitate formed in a precipitin reaction is unsettled. Some workers share the view expressed by Dean and Webb (1926) that the amount of precipitate probably depends on the relative proportions of colloid and electrolytes, the dilution, rate of flocculation and presence of precipitable protein; while others assume that the amount of precipitate is independent of these factors. The point has considerable importance in relation to the nature of the reaction taking place. In recent papers (Marrack and Smith, 1930; Smith and Marrack, 1930) we have shown that the amount of precipitate obtained from a balanced mixture of diphtheria toxin and antitoxin is not affected by the factors suggested by Dean and Webb. In this paper we have continued the investigation of this point in connection with a precipitin reaction with horse-serum pseudo-globulin as the antigen.

METHODS.

The globulin was precipitated from horse-serum by half saturation with $(NH_4)_2SO_4$. The precipitate was dissolved, re-precipitated twice, and dialysed against distilled water for two weeks. The unprecipitated pseudo-globulin was used as antigen.

Rabbits were immunized, and the optimum proportions of antigen and antibody found according to the methods of Dean and Webb (1926). For convenience the amount of antibody equivalent to 1γ (= 1/1000 mgm.) of

antigen was called a unit ; this could be measured with an error of less than 10 per cent. The sera employed and number of units per 1 c.c. were as follows : 284 C (1140 units), 297 B (3200 units), 298 B (4000 units), 298 c (1340 units), and 284 D (2150 units) which had been dialysed against 0·9 per cent. NaCl solution to remove phosphates and sulphates, in order to avoid precipitation of barium salts in experiments 32, 34 and 36. From serum 297 B a solution of pseudoglobulin 297 BP was prepared, by half saturation with $(NH_4)_2SO_4$ and dialysis against distilled water, which contained 790 units per 1 c.c. The euglobulin which precipitated on dialysis contained the greater part of the antibody.

In the actual experiments antigen and antibody were mixed cold, to avoid the possible formation of irreversible precipitates before proper mixing had taken place, and then heated for 90 minutes at 37° C. or 43° C., or frozen solid, and left in the ice-chest overnight. When the mixtures were frozen solid a concentrated mixture of antigen and antibody was obtained (*cf.* Freundlich, 1922) and flocculation was very complete.

The precipitates formed were centrifuged off and washed three times (except in the experiments on the effect of washing) with 0·9 per cent. NaCl solution to which one-tenth its volume of M/15 phosphate mixture was added, giving a pH of approximately 6·7. The precipitate was dissolved in N/500 HCl. Nitrogen was estimated colorimetrically by direct Nesslerization.

In the Table the ratio antibody/antigen is given as 1/1 when the antibody and antigen were mixed in optimum proportions. The limits of the zone of precipitation were approached when this ratio equalled 5/1 or 0·27/1.

RESULTS.

Effect of Washing.

Experiments 24, 25, 26 and 27 show the effect of washing with unbuffered 0·9 per cent. NaCl solution. The loss is of about the same order as that found by Wu, Sah and Li (1929). There is no great difference in the loss from washing the precipitate from a mixture of antibody and antigen in optimum proportions and from a mixture with excess of antigen.

When a buffered washing solution was used (Experiments 28, 29, 1 and 2) the loss was definitely less. It was not so great that variations in this loss would make an appreciable difference to our results, and we have found good agreement in duplicate experiments.

Effect of Dilution.

When a mixture of antibody and antigen in optimum proportions or with antibody in excess was diluted with sodium chloride solution the amount of precipitate obtained was reduced (Experiments 3, 4, 7, 18 and 22). When the flocculation took place at 43° C. (Experiment 9) the effect of dilution was less than at 37° C. Since in both cases the precipitate and supernatant fluids stood overnight at about 0° C. this difference appears to be due largely to more complete flocculation of an insoluble compound at 43° C., and not to the greater insolubility of the compound at the higher temperature.

Experiment number.	Antibody.	Volume of antibody (c.c.).	Ratio antibody/ antigen.	Total volume of mixture (c.c.).	Temperature of flocculation.	Precipitate total (mgm.).	Precipitate γ per unit.	
24	298 C	1	1/1	2·1	Frozen	7·4	5·5	Washed 3 times with 10 c.c. unbuffered saline.
25	,,	,,	,,	,,	,,	6·6	4·95	,, 6 ,, ,, ,, ,,
26	,,	,,	0·28/1	2·3	,,	6·1	4·55	,, 3 ,, ,, ,, ,,
27	,,	,,	,,	,,	,,	5·0	3·7	,, 6 ,, ,, ,, ,,
29	,,	,,	1/1	2·1	,,	7·5	5·6	,, 3 ,, ,, buffered ,,
28	,,	,,	,,	,,	,,	7·15	5·35	,, 6 ,, ,, ,, ,,
1	284 C	,,	3/2	4·8	,,	4·8	4·2	,, 3 ,, ,, ,, ,,
2	,,	,,	,,	,,	,,	4·8	4·2	,, 4 ,, ,, ,, ,,
3	,,	,,	1/1	5·2	37°	5·5	4·8	
4	,,	,,	,,	,,	Frozen	5·6	4·9	
7	,,	,,	,,	27·2	37°	4·6	4·05	
9	,,	,,	,,	22·2	43°	5·0	4·4	
10	,,	,,	,,	,,	Frozen	5·25	4·6	
18	297 B	0·7	5/1	3·2	,,	4·25	1·9	
20	,,	,,	,,	26·4	37°	3·25	1·45	
19	,,	,,	0·34/1	3·5	Frozen	12·5	5·5	
21	,,	0·6	,,	28·4	37°	4·0	2·08	
15	,,	1·0	1/1	6·5	Frozen	15·7	4·9	
22	,,	0·5	,,	27·2	37°	6·4	4·0	
8	284 C	1	,,	23·2	,,	4·5	3·95	pH 6·6.
31	284 D	,,	2·2/1	26	43°	5·15	2·4	
32	,,	,,	,,	,,	,,	5·0	2·3	Mixture contained 0·1 N. $BaCl_2$.
33	,,	,,	0·55/1	,,	,,	7·6	3·5	
34	,,	,,	,,	,,	,,	7·6	3·5	Mixture contained 0·1 N. $BaCl_2$.
35	,,	,,	0·27/1	,,	,,	3·9	1·8	
36	,,	,,	,,	,,	,,	5·05	2·35	Mixture contained 0·1 N. $BaCl_2$.
16	297 BP	,,	1/1	2·9	Frozen	3·3	4·2	,, ,, 0·9 per cent. NaCl.
17	,,	,,	,,	2·8	37°	3·2	4·05	,, ,, 0·33 per cent. NaCl.
13	,,	,,	,,	2·9	Frozen	3·3	4·2	1 c.c. guinea-pig euglobulin added.
5	284 C	,,	,,	2·2	37°	5·6	4·9	
6	,,	,,	,,	5·2	,,	5·5	4·8	3 c.c. fresh guinea-pig serum added.
37	284 D	,,	0·30/1	8·2	43°	7·75	3·6	
38	,,	,,	,,	,,	,,	7·8	,,	5 c.c. human serum added.

However even when the mixtures were frozen (Experiments 4 and 10) less precipitate was obtained from the large volume than from the small volume. In this case the actual combination of antibody and antigen occurs in a very small volume ; the loss of precipitate must have been due to resolution after thawing. These experiments and those on the effect of washing suggest that the precipitate is slightly soluble (about 0·35 mgm. in 20 c.c.), or that the antigen-antibody compound dissociates slightly.

When antigen was in excess (Experiments 19 and 21) the effect of dilution was very much greater. We have seen, however, that the effect of washing did not indicate that such a precipitate was much more soluble than that from a balanced mixture. This suggests that this loss in the larger volume is due to incomplete flocculation of an insoluble compound ; this is also indicated by the effect of the addition of $BaCl_2$ described later.

Effect of Electrolytes and pH.

Experiments 7 and 8 show that a change of pH from 8·0 to 6·6 had no effect. When the volume was small the reduction of the salt concentration to 0·33 per cent. had no effect (Experiments 16 and 17). When the ratio antibody/antigen was in approximately optimum proportions, the addition of $BaCL_2$ up to 0·1 N had no effect (Experiments 31, 32, 33, 34), but when antigen was in excess approaching the limit of the flocculation zone, considerably more precipitate was obtained when $BaCl_2$ was added (Experiments 35 and 36). Presumably the bivalent kation neutralized the charge on the insoluble antigen-antibody compound and produced more complete flocculation.

Effect of Temperature.

It appears from Experiments 3 and 4 that when the volume is small the effect of the temperature at which flocculation occurs is negligible.

Effect of Non-Specfic Proteins.

Experiments 5, 6, 16, 13 and 37, 38 show that the addition of non-specific proteins had no effect even when the antigen was in great excess. The solution of pseudo-globulin used in Experiments 16 and 13 contained only 9 mgm. of protein in 1 c.c. On the addition of 0·79 mgm. (the optimum amount) of antigen, 3·3 mgm. of precipitate formed. If the antigen was wholly precipitated, 2·5 mgm. out of the 9 mgm. of protein in 1 c.c. of the antibody were precipitated. The effect of a solution of guinea-pig euglobulin was tested owing to the work of Dean (1912) and the solution was made according to his directions, but only made up to twice the volume of the original guinea-pig serum. It is probable that the effect observed by Dean was similar to that we have found with $BaCl_2$, as this euglobulin also assists flocculation.

In Experiment 6 the complement was completely removed from the supernatant fluid, but the amount of protein in the precipitate unaffected. A similar result was obtained by Lampl and Landsteiner (1917) in experiments designed for a somewhat different object.

Composition of the Precipitate.

Mixtures containing optimum proportions of antigen and antibody gave from 4·2 to 5·6γ of protein in the precipitate for 1γ of antigen. If almost all the antigen is precipitated, this means that some four-fifths of the precipitate comes from the serum.

According to Douglas and Dudley (quoted by Hartley, 1925), 25 per cent. of the precipitate from a precipitin reaction was soluble in fat solvents; our results do not confirm this. The precipitate from 5 c.c. of serum 298 C was dissolved in 0·9 c.c. of 0·04 N.HCl, added by drops to a mixture of three parts of alcohol and one of ether, sufficient 0·04 N. NaOH added to bring the pH to approximately the iso-electric point of the proteins and the mixture brought to boiling on a water-bath. After boiling the mixture was made up to 50 c.c. with alcohol-ether mixture. The filtrate contained only 6·4 mgm. of solid which consisted almost entirely of sodium chloride; no material soluble in ether or hot chloroform was detected in it, nor in the alcohol washings of the precipitate. The precipitate washed and dried weighed 37·2 mgm. and contained 15·5 per cent. of nitrogen. The globulin-antiglobulin precipitate therefore appears to consist wholly of protein.

Ultra-violet Absorption.

We found (Marrack and Smith, 1930) that the ultra-violet absorption of solutions of diptheria toxin-antitoxin floccules was the same as that of horse-serum pseudoglobulin, the specific absorption at Å. 2804 being 12. We have not found the specific extinction coefficient of these globulin-antiglobulin precipitates at this wave-length as constant as that of the toxin-antitoxin floccules. In one experiment, in which the protein in the precipitate obtained from serum 298 B was estimated by a macro-Kjeldehl method, the specific absorption was 14·3. This was the average figure found in other experiments in which the protein was estimated by the colorimetric method. However, the specific extinction coefficient of solutions of pseudo-globulin from normal rabbit-serum appears more variable than that of horse-serum pseudo-globulin; that of one sample that we have prepared has been as high as 15. We propose to study this question more fully.

DISCUSSION.

These experiments show that the amount of precipitate obtained when antigen (horse-serum pseudo-globulin) and antibody (rabbit serum) were mixed in optimum proportions was relatively independent of the condition under which precipitation took place. The addition of non-specific proteins had no effect, and the effects of variation in the concentration of electrolytes and temperature at which the flocculation took place were only appreciable when the mixture of antigen and antibody was much diluted. In our previous papers on diphtheria toxin-antitoxin floccules (Marrack and Smith, 1930; Smith and Marrack, 1930), we maintained that the fact that the amount of floccules was independent of conditions, particularly of the presence of non-specific protein, indicated that the floccules consisted only of the specific reacting substances. The similar results obtained with a different type of immunity reaction greatly strengthen this point of view.

If our interpretation of the effect of $BaCl_2$ on the amount of precipitate obtained when antigen was in excess is correct, it is impossible to regard the supernatant fluid as a saturated solution of the antigen-antibody compound and apply the laws of mass action on this supposition. It is true that the great effect of dilution, when antigen is in excess, would be explained by the laws of mass action (supposing a compound AG_n to be formed), but in view of the effect of $BaCl_2$ we do not think this is the correct explanation.

CONCLUSIONS.

1. The compound of horse-serum pseudo-globulin and its antibody is slightly soluble in 0·9 per cent. NaCl solution.

2. The amount of precipitate obtained from mixtures of antigen and antibody in optimum proportions is not affected by moderate variations in the concentration of electrolytes. When antigen is in excess and the volume large, the amount is greatly increased by the addition of 0·1 N $BaCl_2$. This we consider to be due to the neutralization of the negative charge of an insoluble suspension.

3. The amount of precipitate is unaffected by the addition of non-specific proteins.

4. The precipitate is composed of protein; no substances soluble in fat solvents were detected.

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THE RELATION BETWEEN FOWL-POX (EPITHELIOMA CONTAGIOSUM) AND AVIAN DIPHTHERIA.

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THE individuality or identity of the diseases known as fowl-pox and avian diphtheria has been and still is the subject of controversy. The literature contains much conflicting evidence tending to support one or the other view, and the interchangeable use of the two terms is convincing proof that the results of the investigations reported thus far have not been conclusive.

Carnwath (1908) reports studies of a disease the clinical aspect of which was that of avian diphtheria. Except in one case the lesions were localized entirely in the mouth and respiratory mucous membrane, with complete absence of skin-lesions. However, inoculation of material from these lesions on the combs of normal fowls resulted in typical fowl-pox lesions. No histological studies were made. This author doubts whether pure primary diphtheria exists, and recommends the inoculation of the diphtheritic membrane into normal fowls to demonstrate the presence of the virus. Uhlenhuth and Manteufel (1910) were also able to demonstrate the virus of fowl-pox in diphtheritic membranes. They admit, however, that other agents may cause the clinical picture of avian diphtheria. Schmid (1909), on the other hand, tested the material from ten cases of avian diphtheria and was able to demonstrate the virus of fowl-pox in only 3 of the 10 cases tested. Fally (1908) also failed to demonstrate the virus of fowl-pox in the cases of avian diphtheria studied by him.

Other authors differentiate clearly between avian diphtheria and fowl-pox, and report the isolation from diphtheritic membranes of bacteria which could reproduce the disease. Loeffler (1898) isolated a Gram-negative organism which he called *B. diphtheriæ avium*. Loir and Ducloux (1894) also isolated a Gram-negative bacillus from the spleen of chickens dead from diphtheria, with which they were able to reproduce the disease. Streit (1904) reports an extensive epidemic of avian diphtheria without a single case of fowl-pox. Bordet and Fally (1910) also considered avian diphtheria entirely distinct from fowl-pox, and isolated a Gram-negative bacillus from cases of avian diphtheria which they believed to be the ætiological agent.

It is apparent from this brief summary that there is no clear differentiation of the diseases in question. It appears that there are cases with diphtheritic lesions in the oral and pharyngeal mucosa in which the virus of fowl-pox is regularly present and others in which the virus is absent. It is by no means clear, however, whether the lesions in these cases are identical, or whether the term " diphtheritic membrane " covers a variety of membranes of different character and ætiology. It is well known that both in experimental and spontaneous fowl-pox, specific lesions may occur in the oral and pharyngeal mucous membrane along with the cutaneous lesions (Bollinger, 1873 ; Burnet, 1906 ; Lipschuetz, 1908 ; Goodpasture, 1928). On the other hand, there are records of epidemics of avian diphtheria in which the nasal and oral passages

PLATE I.

FIG. 1.—Early fowl-pox lesion on tongue.

FIG. 2.—Inoculated comb from tongue lesion (fig. 1). Typical fowl-pox lesion.

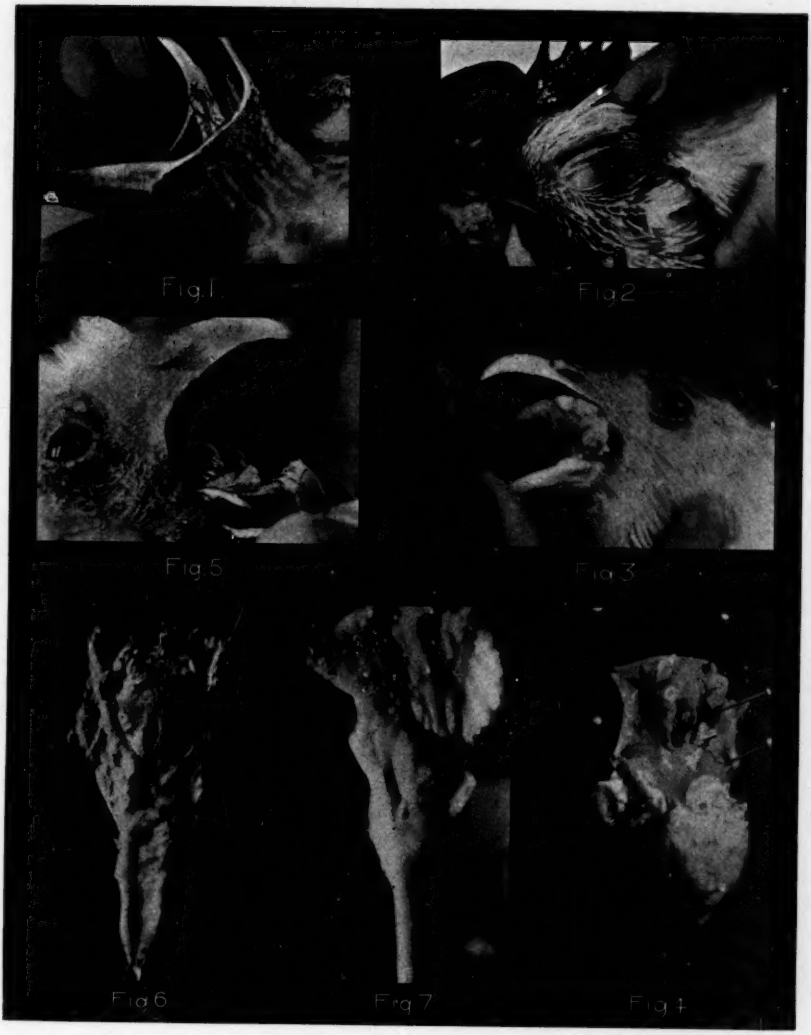
FIG. 3.—Late stage of fowl-pox infection on tongue. By extension the tumour-like growth has now enveloped the entire tongue.

FIG. 4.—Fowl-pox. Tongue shown in fig. 3 after removal from chicken.

FIG. 5.—Diphtheria lesion. Note irregular strand-like patches on various parts of tongue. No fowl-pox could be produced with material from the plaques.

FIG. 6.—Tongue of chicken shown in fig. 5 after removal. Contrast appearance with fowl-pox tongue shown in fig. 4.

FIG. 7.—Tongue of chicken showing multiple abscesses typical of avitaminosis.



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are involved, without the presence of fowl-pox virus or the cutaneous lesions characteristic of fowl-pox. In the course of our studies (1929, 1930) on fowl-pox we have had occasion to note distinct cases of diphtheria as well as mixed cases of diphtheria and fowl-pox. We have therefore utilized the opportunity to study the nature of these infections, their identity or distinctiveness. The present paper presents our observations on this subject.

METHODS AND MATERIAL STUDIED.

Ordinarily the identity or individuality of two apparently distinct diseases might easily be determined in a number of ways. The simplest procedure would probably be to ascertain the cross immunological relationships. In chicken diphtheria this procedure does not yield conclusive results, because it is not transmissible with the same degree of certainty as fowl-pox. Whereas fowl-pox can be transmitted to non-immune chickens, *i. e.* birds which have not been exposed to the infection, with absolute certainty, this is not always the case with avian diphtheria. In our experience transmission of the latter disease gave irregular results. Moreover, at times clinical avian diphtheria arose spontaneously in either normal or fowl-pox-infected chickens. Cross-immunity experiments did not, therefore, yield conclusive results.

In our studies, therefore, we resorted to a number of methods. In so far as possible cross-immunological relationships were observed in spontaneous as well as experimental infections. More satisfactory results were obtained by ascertaining the presence of fowl-pox virus in diphtheritic lesions, as suggested by Carnwath (1908). Finally conclusive data were obtained by a histological study of the lesions.

OCCURRENCE OF DIPHTHERIA IN CHICKENS IMMUNE AGAINST FOWL-POX.

The following are a series of selected protocols showing the spontaneous development of diphtheria in fowls which had recovered from an infection of contagious epithelioma :

1. Chicken 18 :—May 4th, inoculated with fowl-pox ; lesion positive May 13th. Lesions receding but still present on comb on July 13th, when chicken died from heavy diphtheria infection. (Material preserved in glycerine.)

2. Chicken 23 :—Inoculated with fowl-pox on July 7th. Positive July 15th. August 27th, after recovery from fowl-pox, the chicken along with others not inoculated died from typical diphtheria infection, which was then epidemic.

3. Chicken 24 :—Infected with fowl-pox on July 8th. Positive after 5 days. August 28th, normal. September 11th, reinoculated with fowl-pox virus. No lesions developed. On September 20th became ill with diphtheria.

4. Chicken 25 :—Infected July 16th. Positive July 21st and 22nd. August 28th, when the fowl-pox lesions were healed, typical diphtheria infection developed.

5. Chicken 8 :—Infected January 18th. Lesions present on January 28th ; Secondary lesions on February 10th. Lesions had receded on March 1st, when diphtheria infection developed, resulting in death.

It is apparent from these protocols that a spontaneous diphtheritic infection may occur in chickens already partially or wholly immune to fowl-pox. Although fowl-pox immunity is not always as solid as that in virus diseases of other animals, an immunity is established, and reinoculation after recovery from an infection or even after vaccination results only in an abortive lesion, if any. Fowl-pox and avian diphtheria may therefore be considered immunologically distinct diseases.

INOCULATION OF DIPHTHERITIC MEMBRANES.

The inoculations of spontaneous diphtheritic membranes on the combs and wattles of susceptible chickens confirmed the results obtained by Schmid (1909). Some membranes yielded positive pox infections, others remained entirely negative. These results also indicated that we were dealing with two types of infection, which occurred either independently or simultaneously in the same fowl. The following are some selected protocols :

1. Two infected chickens were brought to the laboratory. Chicken 32 had fowl-pox lesions on both feet and a yellowish plaque on the border of the left side of the tongue. Chicken 35 had a whitish plaque on the hind part of the tongue and on the pharynx.

Chickens 28 and 29 were inoculated at the same time with the plaque material from fowls 32 and 35 respectively. The plaque from 32 was inoculated on the left combs and that of 35 on the left wattles. The former were positive after 4 days and typical fowl-pox lesions developed ; the latter remained negative.

2. Chicken 30 :—(*a*) September 18th, inoculated with plaque from chicken 32 under tongue. Lesion appeared on September 22nd and continued growing until the whole tongue was involved (see Plate I, fig. 3).

(*b*) On September 24th both eyes showed diphtheritic infection with thick, whitish, fibrinous masses. The nasal sinuses were also involved. This material was inoculated on the comb and tongue of chicken 39. On September 27th comb and tongue negative ; both eyes swollen. On September 29th whitish patches on hind part of tongue, eyes closed and filled with pus ; diphtheria positive, fowl-pox negative.

(*c*) Material from the tongue lesion of chicken 30 was inoculated on the comb of 30*a* and 40 (see below), and resulted in typical fowl-pox lesions.

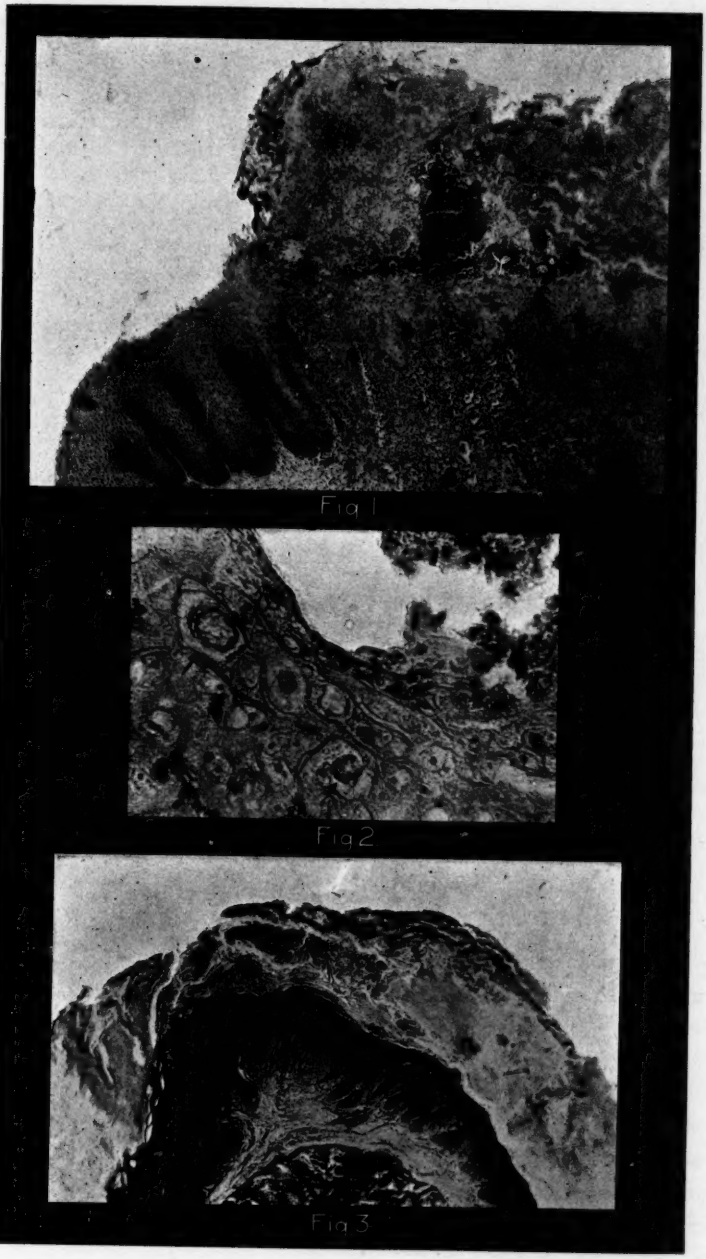
3. (*a*) Membrane from spontaneous diphtheritic lesions (chicken 40) was inoculated on the left wattle, left side of the tongue and left eye of 8 chickens.

PLATE II.

FIG. 1.—Section of fowl-pox lesion on tongue corresponding to fig. 1, Plate I. Fixation in Zenker, stained with hæmatoxylin-eosin ; × 80. Note extensive necrosis of epithelium and infiltration.

FIG. 2.—Same as fig. 1, stained with Giemsa ; × 1000. Note enlarged epithelial cells with typical inclusion bodies indicated by arrows.

FIG. 3.—Section of diphtheria membrane. Fixed in formalin, stained with Giemsa ; × 80. The epithelial tissue is practically intact ; the membrane covering the surface consists of a mass of bacteria enmeshed in a muco-fibrinous network. Contrast this picture with that shown in fig. 1. No inclusion bodies were found.



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Next day a small plaque appeared on the tongue at the point of inoculation, but this disappeared two days later and no further lesions were noted.

(*b*) At the same time this chicken (40), having a diphtheria infection, was inoculated on the left side of the comb and tongue with material from the tongue lesion of chicken 30. After 4 days a plaque appeared on the left side of the tongue ; after 7 days vesicles developed on the comb, the lesion on the left side of tongue growing.

4. Inoculation of diphtheria into chicken infected with fowl-pox : Chicken A bought in the market on October 15th. After two weeks in the animal-house this chicken developed typical diphtheria ; no fowl-pox lesions were noted. The tongue and upper and lower palates were coated with irregular patches of whitish caseous material, the left eyelid was swollen and the eye closed ; the sinus was filled with a whitish pus. This chicken was killed, the tongue removed for photographs (see Plate I, fig. 6). The nasal passage on left side was obstructed by a whitish, cheesy pus ; the larynx also was covered with the same material, and there were irregular patches in the pharynx.

Material from this chicken was inoculated into chickens 43 and 44 infected with fowl-pox, but free from diphtheria. (*a*) Chicken 43 was inoculated with fowl-pox on the left comb on October 25th. On November 1st when fowl-pox was developing it was inoculated as follows :

a. In the right eye, fluid from infected eye.

b. On right comb, fluid from infected eye.

c. Under right side of tongue and on right comb, caseous diphtheritic membrane from tongue.

November 6th : The pox lesion scaling ; other inoculations negative.

November 14th : Left eye closed, full of pus and serous fluid.

November 19th : Dead. Autopsy : left eye blocked with solid mass of white cheesy material. Both nasal passages blocked completely with same material. Tongue clear and pharynx clear.

(*b*) Chicken 44 :—Fowl-pox infection, October 30th. Diphtheria inoculation as in chicken 43 on November 1st. November 14th : Fowl-pox lesion developing ; diphtheria inoculations negative. November 24th : Fowl-pox lesions healing ; no signs of diphtheria. Chicken recovered. No signs of diphtheria.

THE SIMULTANEOUS OCCURRENCE OF FOWL-POX AND DIPHTHERIA.

The following experiments bearing on the simultaneous occurrence of fowl-pox and diphtheria are also of interest : Chicken 18 had a fowl-pox lesion on the comb and a diphtheritic infection in eyes and oral cavity. Transfers were made from both lesions :—

1. Chicken 21 :—On right comb pox virus from chicken 18 ; on left comb and into both eyes and nose diphtheritic material from chicken 18. Fowl-pox inoculation positive, others negative.

2. Chicken 22 :—July 3rd, right comb and pharynx inoculated with diphtheritic material, and left wattle with eye material from chicken 18.

On July 10th one vesicle on right comb ; others negative.

Note.—Apparently in mixed infections small amounts of virus may at times be demonstrated in the diptheritic material ; the concentration of virus

is, however, very low, in contradistinction to the massive amounts present in diphtheritic fowl-pox lesions.

3. Chicken 26 :—August 2nd right comb, eye and nose inoculated with glycerinated diphtheria material from chicken 18 ; left comb and eye with glycerinated fowl-pox material from chicken 18.

On August 7th fowl-pox lesions on left comb. Rest negative. On August 28th fowl-pox healed ; no diphtheria.

HISTOLOGICAL STUDIES.

In the course of these studies it became more and more apparent that we were dealing with three distinct types of infection of the oral cavity, not always readily distinguishable from one another. These conditions differed primarily in the character of the lesions and quality and texture of the membranes. The most characteristic condition was that due to fowl-pox virus. In these cases the diphtheritic lesions were almost invariably associated with cutaneous infection. The membrane was circumscribed, leathery in consistency, yellowish in colour and adherent to the mucous membranes. They had more the appearance of a growth than a membrane. Most often the mucosa of the mouth as well as the tongue was involved, more infrequently the pharynx ; where the pharynx was involved the lesion had the appearance of an ulcer. In the cases of fowl-pox the virus could invariably be demonstrated by transfer to the comb or wattle of a normal fowl.

The second condition which we consider as true avian diphtheria differs essentially from the one described above. Cutaneous fowl-pox lesions are as a rule absent. There is usually, but not always, an involvement of one or both eyes as well as the nasal sinuses. But the most characteristic point of differentiation is the character of the membrane. The membrane is whitish, fibrinous, non-adherent, and covers the tongue and mucosa of mouth, pharynx and œsophagus, sometimes extending into the crop ; it is not discrete and well defined as in fowl-pox, but rather ragged and irregular.

Another difference between these two affections is that the membrane found in fowl-pox, when transferred to the tongue of another chicken, develops slowly, progressively involving the whole tongue by extension much like a cancerous growth. Death may result from occlusion of the larynx. The diphtheritic membrane may yield negative results on inoculation, but when positive the nasal passages are first involved. The membranes when formed are irregular in distribution, the œsophagus as well as the oral mucosa is involved, and death may often result either from an obstruction of the trachea, or from a general inflammation of the mucous membrane of mouth, pharynx and crop.

The third condition is not always easily distinguished from the second, but has some points of difference. It is due to an avitaminosis and not to an infection. Like in diphtheria there is an involvement of the eyes and sinuses, the lids being swollen and the sinuses filled with a whitish cheesy pus. Unlike diphtheria there is no membrane, but instead a characteristic affection of the mucosa of the pharynx and œsophagus. The œsophagus is covered with many pin-point abscesses, due to an infection of the numerous glands (Plate I, fig. 7).

This disease is not transmissible, and can be cured if treated early enough by addition of green food to the regular diet.

The histological study of the two types of diphtheritic lesions confirms the macroscopic difference noted. The membrane due to fowl-pox virus actually penetrates the epithelial layer. The virus attacks the epithelial layer of the tongue causing extensive necrosis. The growth is of the same nature as that on the comb ; there is first an enlargement of the cells, and characteristic fowl-pox inclusion bodies are present in the cells in the affected areas. After a while the cells disintegrate and the necrosis is followed by an invasion of bacteria (Plate II, figs. 1 and 2). In contrast with this the diphtheritic membrane is superficial, attacks only the surface layer of epithelial cells which do not contain inclusion bodies, and consists chiefly of masses of bacteria entangled in a fibrinous network (Plate II, fig. 3). In both cases there is extensive infiltration, probably due to the bacterial invasion.

The accompanying plates show the macroscopic and microscopic appearance of the lesions noted in the three types of affection studied.

DISCUSSION.

The problem of the identity or distinctiveness of fowl-pox and avian diphtheria is one of considerable practical importance. The control of these diseases depends in a large measure on a knowledge of their ætiology and mode of transmission. Moreover, recent studies indicate that chickens can be effectively protected against fowl-pox by vaccination with a proper vaccine.

It seems to us that our results warrant the conclusion that we are concerned with two distinct disease entities, one caused by a specific ultramicroscopic virus, the other as yet of uncertain ætiology. The two types of diphtheritic affections may occur simultaneously or independently. The frequent association of the two diseases may be due to a superinfection resulting from a lowering of the resistance of the fowl by the fowl-pox virus. Secondary infection associated with or following virus infections is one of the characteristic features of a number of virus diseases, and it may well be that the fowl-pox infection is a precursor of invasion by one or more types of bacteria.

CONCLUSIONS.

1. Fowl-pox or contagious epithelioma and avian diphtheria are two distinct disease entities which may occur simultaneously or independently. There is no cross-immunity between these affections.

2. Fowl-pox virus may attack the tongue or oral mucosa with the production of ulcerative membranes. The membrane in this case is adherent, causes bleeding on removal, is leathery in consistency and circumscribed. Microscopically it is characterized by extensive necrosis of the epithelial tissue, infiltration, and the presence of typical inclusion bodies.

3. The diphtheritic membranes are irregular whitish or yellowish patches, cheesy in consistency, only slightly adherent. They do not contain fowl-pox virus. Histologically they appear as superimposed layers on the epithelium, which is only slightly injured. There is infiltration but little, if any, necrosis of tissue and no inclusion bodies are found.

4. The type of lesions which we found due to avitaminosis is distinctly different. Macroscopically they have the appearance of whitish pustules scattered over the whole of the mucous surface of the pharynx. Microscopically the lesion is shown to be confined to the glands.

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RECOVERY OF FOWL-POX VIRUS FROM VACCINES BY CATAPHORESIS.

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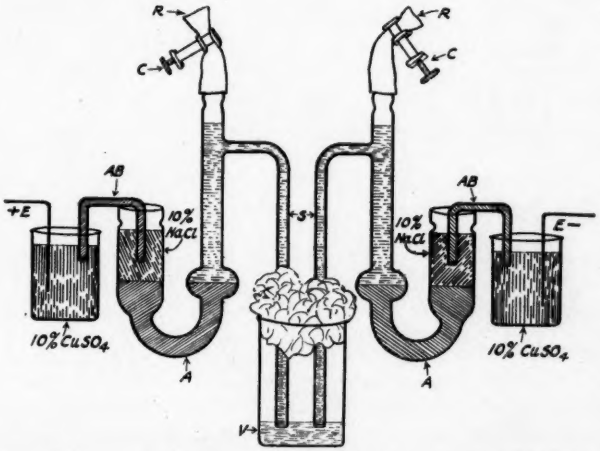
A NUMBER of investigators have studied the nature of the electric charge of filterable viruses. Angerer (1924), Koch (1926) and Todd (1927) conducted cataphoresis experiments with bacteriophage. Angerer found that the bacteriophage wandered to the anode. Koch, working with a Shiga bacillus bacteriophage, reported that it moved to the cathode. Todd, on the other hand, also working with Shiga bacteriophage, found that in solutions having a pH range of 3·6 to 7·6 the migration was in the direction of the anode.

The results thus far reported with ultramicroscopic viruses are also variable. Olitsky and Boez (1927) studied the nature of the charge of foot and mouth disease virus, and found that it carried a positive charge on the acid side of pH 8·0. Douglas and Smith (1928), working with vaccinia virus, found that it carried a negative charge between pH 5·5 to pH 8·4. Subsequently Olitsky and Long (1929, *a*), (1929, *b*) showed that virus can be separated from its antibody by cataphoresis, and that this procedure could be employed to recover virus from tissues of immune animals.

Previous studies (Kligler, 1930) have shown that effective immunization with fowl-pox vaccines could be obtained only when live virus was present, and that in the absence of live virus the vaccine was not effective. The work of Olitsky and Long suggested that it might be possible by cataphoresis to demonstrate active virus in vaccines which still produced immunity without causing typical lesions. The object of this paper is to report the results of experiments in which fowl-pox virus was recovered from effective vaccines by cataphoresis.

TECHNIQUE.

The apparatus used is shown in the accompanying drawing. It is an adaptation of the system described by Michaelis. The vessels were clamped to



R = rubber tubing; C = clamp; AB = agar bridge; E = copper electrodes; S = side arms of tubes filled with buffer solution; A = 3 per cent. agar in 1 per cent. saline solution; V = virus suspension.

a stand which was so fitted that four tests could be run simultaneously. The long tubes and side arms were filled completely with the desired buffer solution, which was kept in the side arms by tightly clamping the arms of the tubes at C. To facilitate the flow of current the buffer solutions were diluted with 0·5 per cent. NaCl solution. To prevent diffusion, the bent parts of the tubes were filled with a 3 per cent. agar solution in 1·0 per cent. NaCl. The upper parts of the bent tubes were filled with 10 per cent. NaCl solution, and an agar bridge connected them with the copper-sulphate solution. Non-polarizable copper electrodes were used. The side arms were immersed in a sterile vial containing the vaccine suspension. The apparatus was attached to the main 220 volt d.c. and allowed to run three hours. With this arrangement there was a slight rise in the temperature of the side arms, but no overheating occurred.

The apparatus was cleaned, the open ends plugged with cotton and sterilized

by dry heat. Before using, the curved parts of tubes were filled with the salt agar mixture. After the agar had set sterile buffer solution was added, care being taken that the side arms (s) were completely filled. The side arms were then immersed in the vial containing the test fluid. The conducting solutions and electrodes were then put in place and the plug put in the wall socket.

After 3 hours the system was disconnected. The anode tube was then invariably turbid. The apparatus was lifted gently out of the vial, and each end dried with sterile filter-paper.

By manipulating the clamps, about half the fluid from each of the side arms was run off separately into small sterile test-tubes. Inoculations were made by scarifying the comb or wattle of a susceptible chicken and rubbing in one or two drops of the fluid to be tested.

RESULTS.

For the purpose of these experiments three vaccines were used. Two were phenolized vaccines prepared in the manner described in the previous paper (1930), and one was a dried commercial vaccine known as the de Blieck vaccine. On direct scarification large doses of the phenol vaccine gave single discrete vesicles after 9 days' incubation, while the de Blieck vaccine at times gave positive results after much delayed incubation (10–16 days), though at other times the results were negative. In all cases young susceptible Leghorns were used.

By means of cataphoresis it was possible to obtain sufficient concentration of virus from each of the three vaccines to give positive lesions in five days. The virus was always recovered at the anode, while the material taken at the cathode was negative. In this respect the results differ from those reported by Natarjan and Hyde (1930), who found that the fowl-pox virus was amphoteric. The following experiment is illustrative :

The experiment was conducted with M/15 solutions of phosphate buffers diluted with 2 parts saline solution at pH 7·0. To an equal amount of the diluted buffer solution 5 c.c. of the concentrated phenol vaccines were added. The de Blieck vaccine, which is put up in the form of a powder, was first suspended by thoroughly triturating 0·1 gm. of the vaccine in 5·0 c.c. of a physiological saline solution ; this suspension was centrifugalized at low speed for 5 minutes, and the supernatant fluid added to the buffer solution in the same manner as the phenolized vaccines. The cataphoresis lasted 3 hours. The same quantity of material was removed from each side arm of the apparatus and inoculated on the comb and wattle of a young chicken. All the tests were conducted simultaneously and inoculated on the same chicken. The results are summarized in Table I :

TABLE I.

Vaccine.	Material and site of inoculation.		Results.	
	Anode.	Cathode.	5 days.	12 days.
De Blieck	Left comb front	Left comb rear	Front +	Front +; rear −
Phenol A	Right ,,	Right ,,	,, +	,, +; ,, −
,, B	Left wattle front	Left wattle rear	,,	,, +; ,, −
Virus control	Right ,,	Right ,,	,, +	,, +; ,, −

The experiment was repeated with the same results. In all cases, including the control, virus was recovered at the anode. It is noteworthy that the de Blieck vaccine, which is considered one of the most effective on the market, as well as the phenolized vaccines, which in our experiments proved equally effective, contained live virus.

CONCLUSION.

By cataphoresis it is possible to recover at the anode active virus from phenolized and dried fowl-pox vaccines suspended in phosphate buffer solutions at pH 7·0.

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RESISTANCE TO THE GROWTH OF TRANSPLANTABLE TUMOURS. I: THE INFLUENCE OF VITAL STAINING ON INDUCED RESISTANCE.

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IMMUNITY, or natural resistance to tumour transplantation, was first described by Jensen (1903), while Bashford, Murray and Cramer (1908) were the first to show that resistance could be induced by the injection of embryo emulsions, and certain other tissues. Numerous attempts have been made on the basis of these phenomena to devise methods of treatment. They have all met with failure. A review of the literature on the subject has been made recently by Woglom (1929).

Experiments which I have carried out on the effect of dyestuffs and metallic colloids on tumour growth have failed to indicate any definite inhibitory action. Intense "Speicherung" seems rather to break down any resistance that the animals might offer to tumour growth. It therefore seemed probable, that by vital staining, the immunity induced by embryo-tissues to tumour growth might be destroyed. The experiments described in this paper were carried out to investigate such a possibility.

GENERAL SCHEME OF THE EXPERIMENTS.

The general plan of the experiments was as follows : A batch of mice was immunized by injecting embryo skin in the right axilla. These were divided into two groups. The mice of one group were vitally stained a week after the inoculation of the embryo skin. At the end of the second week both groups.

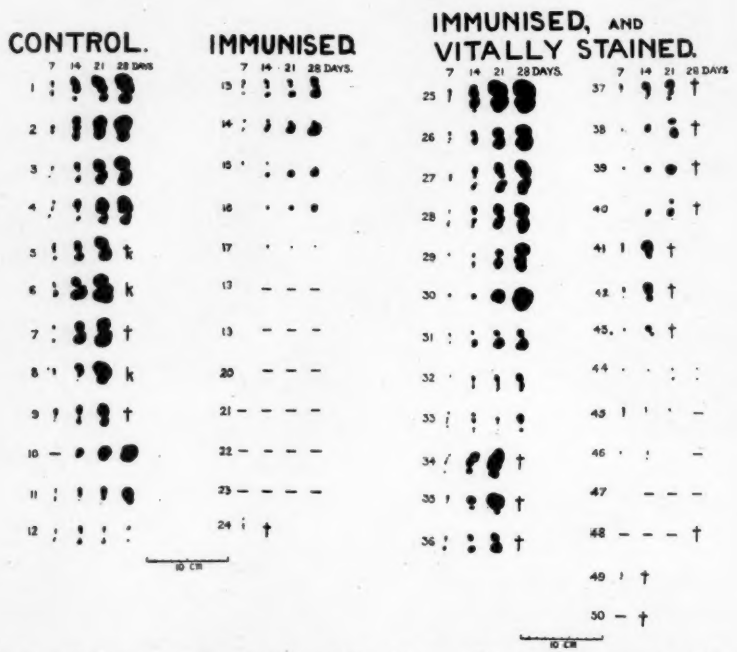

FIG. 1.—Growth of the tumour 63/115A, as indicated by weekly charting from the time of transplantation, in normal mice (control), mice which had been previously immunized by inoculation with embryo skin emulsion, and mice which had been previously immunized and then vitally stained with trypan blue. (K denotes killed; † died.)

together with a set of controls, were inoculated in the left axilla with the transplantable adenocarcinoma 63 of this Laboratory.

PROTOCOLS OF EXPERIMENTS.

Experiment 1. (Fig. 1.)

Immunization.

First day of experiment, 38 mice were injected with 0·025 c.c. of finely minced mouse embryo skin in the right axilla (Nos. 13–50).

Vital Staining.

Twenty-six of the above mice (Nos. 25–50) were injected subcutaneously, on the back, with a 0·5 per cent. solution of trypan blue (Grübler) in distilled water, as follows: 9th day, 0·5 c.c.; 10th day, 0·5 c.c.; 12th day, 0·3 c.c.; and 14th day, 0·5 c.c.

Tumour Transplantation.

Fifteenth day: The 26 immunized and vitally stained mice, together with the other 12 immunized and 12 controls, were transplanted with tumour 63/115A of this laboratory. Tumour emulsion (0·01 c.c.) was injected by syringe into the left axilla of each mouse (Nos. 1–50). The growth of the tumours was charted weekly, the number of days at the top of the chart indicating the number of days after transplantation.

Continuation of Vital Staining of Immunized Mice after Tumour Transplantation.

Twelve mice (Nos. 30, 31, 33, 36 to 40, 42, 44, 47 and 49) received injections of the same solution of trypan blue after transplantation of the tumour as follows: 19th day, 0·4 c.c.; 23rd day, 0·2 c.c.; 26th day, 0·3 c.c.

Taken as a whole the tumours grew slower than those of the other vitally stained and immunized mice. The difference, however, was not sufficiently marked to justify their being separated on the chart.

Result of Experiment.

It will be seen from the chart that of the controls, only one tumour failed to grow. The immunity induced by the embryo skin was not complete. Four tumours out of 12 showed slow growth. Of the immunized and vitally stained mice 19 showed growth out of a possible 26.

Experiment 2. (Fig. 2.)

Immunization.

First day of experiment, 31 mice were injected in the right axilla with 0·05 c.c. of finely minced mouse embryo skin (Nos. 10–40).

Vital Staining.

Twenty-one of the immunized mice (Nos. 20–40) were injected subcutaneously, on the back, with 0·5 c.c. of a 0·5 per cent. solution of trypan blue (Grübler) on the following days from the beginning of the experiment: 8th day, 9th day, 11th day, and 12th day.

Tumour Transplantation.

Fifteenth day: The 21 immunized and vitally stained mice, with the other 10 immunized, and 9 controls, were inoculated on the left side of the body with 0·01 c.c. of an emulsion of tumour 63/120B (Nos. 1–40). The growth of the tumours was charted as in the previous experiment.

Result of Experiment.

The relatively poor growth of the tumour 63 in the control mice indicates that at the time of transplantation this strain was passing through a period of depressed growth. Such a fluctuation in the rate of growth is characteristic of transplantable tumours, and from the observations made in this Laboratory, would appear to be due to some intrinsic variation in the biology of the cancer-cell. In this experiment no tumours grew in the mice which had been

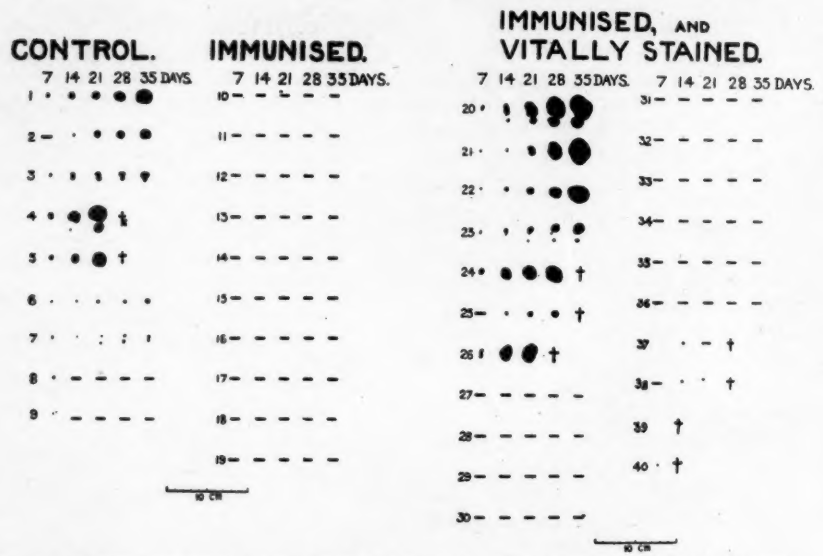

FIG. 2.—Similar experiment to that illustrated in FIG. 1. The tumour transplanted, 63/120B, was at the time passing through a phase of depressed growth. Other differences between the two experiments are described in the protocols.

immunized. Only 7 mice of the 21 immunized and vitally stained developed tumours, but these grew rather faster than the control.

The difference between the results of these two experiments indicates that the lowering, by vital staining, of the resistance to tumour transplantation induced by the inoculation of embryo skin emulsion is independent of the phase of growth of the tumour-cells.

DISCUSSION.

In addition to the two experiments described in detail, a third has been carried out, with similar results. They all indicate that vital staining in some way induces changes in the organism, so that the immunity induced by the inoculation of mouse embryo skin to the transplantation of tumours is broken down.

The tumour-cells fail to stain vitally with trypan blue, but there occurs an intense staining of the cells of the reticulo-endothelial system, and also of the cells of the connective tissues (mesenchyme-cell system). A local cellular reaction in the connective tissues was shown by Da Fano (1912) to occur in the immunizing process. It seems most probable that vital staining modifies this reaction in some manner, so as to affect the general reaction of the host to the inoculated tumour-cells. Since any explanation of this process at present would be purely speculative, further discussion will be deferred until other aspects of the resistance of the host to the growth of transplantable tumours have been considered.

CONCLUSION.

By vital staining with trypan blue it is possible to break down the immunity to tumour transplantation induced by the inoculation of embryo skin emulsion.

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SOME OBSERVATIONS ON PASSIVE IMMUNITY IN THE NORMAL SUBJECT PRODUCED BY ANTIPNEUMOCOCCIC SERA.

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THE recent introduction of concentrated antipneumococcal sera for therapeutic use has attracted special attention to the efficacy of the serum treatment of acute pneumonia. The method of concentration which has been most used is that of Felton (1925,1926), in which the water-insoluble constituents of polyvalent antiserum are redissolved in a solution of sodium chloride, tartaric acid or other solvent. Extensive clinical tests have been made with sera of this type prepared at the Lederle Antitoxin Laboratories, New York, and their therapeutic value and the best methods of administration are still *sub judice* (Cecil and Sutliff, 1928; Park, Bullowa and Rosenbluth, 1928). To obtain more information about the humoral defence mechanism influenced by passive immunization with such anti-sera and the specificity of the effects, a series of

normal healthy persons were given intravenous injections of different antipneumococcal sera, and at varying intervals after the administration their blood was tested for its bactericidal effect on types I, II and III pneumococcus, and their serum for its power of protecting a highly susceptible animal against lethal doses of the three types. It is recognized that antipneumococcal sera exert high protective properties *in vivo* against the specific organisms but the exact mechanism of this reaction is uncertain, though Robertson, Sia and Woo (1924) showed that the serum of animals which have natural or artificial antipneumococcal immunity can opsonize virulent pneumococci so that they are phagocyted by the polymorphs of either immune or susceptible animals. These writers therefore agreed with the opinion that opsonization and phagocytosis are likely to be important in the destruction of pneumococci in infective conditions. The question whether passive immunization would exalt the bactericidal power of the recipient's blood seemed, therefore, of interest.

The results of these passive immunity experiments illustrate the specific immunizing properties of antiserum, and throw some light on the mechanism of antipneumococcal immunity.

BACTERICIDAL POWER OF THE BLOOD.

Six persons were given an intravenous injection of one or another of three preparations of therapeutic antipneumococcal serum, and the defibrinated blood and the serum of each person withdrawn before and on several occasions after the injections were tested for the purposes defined. Two methods of estimating the bactericidal power of the blood have been used. The first method, a technique similar to that used by Mackie and Finkelstein (1931) for testing the bactericidal action of serum, was applied in the experiments Nos. 1–4. The second method, which included certain modifications of the first, was used in the remaining experiments.

First method.—Each specimen of blood was withdrawn with the usual aseptic precautions by superficial veni-puncture, and was thoroughly defibrinated by shaking it for several minutes in a sterile tube or flask containing glass beads. The organisms used were virulent strains of *pneumococcus* of type I, type II and type III. The average lethal dose for mice by intraperitoneal injection of the type I strain was 1 c.c. of a 10^{-6}–10^{-7} dilution in saline of an 18 hours' broth culture; of the type II strain the lethal dose was 1 c.c. of a similar culture diluted 10^{-8}–10^{-9}, and of type III, 1 c.c. of culture diluted 10^{-6}. For the test a series of successive centimal dilutions (from 10^{-2}–10^{-16}) in normal saline of an 18 hours' phosphate-broth culture of each type of organism was prepared, and the test was set up by arranging twenty-one small sterile test-tubes plugged with cotton-wool in three rows of seven tubes each. Into each tube were placed 0·4 c.c. of defibrinated blood and 0·2 c.c. of each dilution of pneumococcal culture. The mixtures were incubated overnight at 37° C. and then the presence or absence of viable organisms was tested by spreading a large standard loopful of the mixture in a single broad stroke on a plate of boiled blood-agar medium. The plate was subdivided by marking with a grease pencil, so that each section accommodated one stroke inoculation. Six or seven strokes were made on a plate of 4 in. diameter. The amount of growth which occurred after 48 hours' incubation was recorded by the figures 4, 3, 2, 1 or 0, according to its amount (see table). At the time that the mixtures of blood and pneumococci were made, each specimen of defibrinated blood was tested for sterility by spreading a loopful of it on a blood-agar plate, which was then incubated at 37° C. for 48 hours.

Second method.—The specimens of blood taken from Subjects 5 and 6 were examined by a method somewhat modified from that already described. The blood was withdrawn by

veni-puncture and defibrinated as before. The strains of the three types of pneumococcus were different, but they were in a similar high degree of virulence (as tested by intraperitoneal injection into mice) to those used in the first method. The cultures used were made in blood-phosphate-broth medium and had been incubated for 18–24 hours. Decimal dilutions in ordinary bouillon were made from these cultures, and for the test the undiluted culture and a range of dilutions from 10^{-1}–10^{-6} was used.

For each specimen of blood the test was set up as before by arranging twenty-one sterile plugged tubes in three rows of seven tubes each. Into each tube was placed 0·4 c.c. of defibrinated blood, and one drop of culture (undiluted or diluted) was added from one of the preparations of type I, II or III pneumococcus. The cotton-wool plug in each tube was fixed in position by pouring hot melted paraffin wax on it and the tubes were placed in an electrical shaking machine, which kept the mixtures of blood and organisms in constant motion while they were incubated overnight at 37° C. The presence or absence of viable organisms at the end of this time was tested for as previously described.

Experiments 1, 2 *and* 3.

In these three cases a different preparation of therapeutic antipneumococcal serum was injected in each and the results are grouped together because the injections were made, and the specimens of blood from all three were set up in the bactericidal test, at the same time as one another.

Subject No. 1 was a man of 19 years. Blood was withdrawn and defibrinated before the injection of serum to serve as the standard of comparison of bactericidal power of the blood. The serum used in this case was Lederle's refined concentrated antipneumococcic serum as issued in 1929. The serum is prepared for therapeutic use in amounts of 10 c.c., and it is claimed that it contains antibodies in high and equal concentrations against types I and II pneumococcus. After a conjunctival test for sensitiveness to horse-serum had proved negative, 10 c.c. of the serum, heated to body temperature, was injected slowly at the rate of about 1 c.c. per minute into a superficial cubital vein. The conjunctival sensitivity test was negative. The time occupied by the injection was 8 minutes. No change in the general condition which could be ascribed to the injection took place during the next 24 hours.

Subject No. 2 was given a similar injection of 20 c.c. of Burroughs Wellcome's therapeutic type I antipneumococcal serum. The injection occupied 12 minutes, and no symptoms or signs of disturbance occurred during the next 24 hours.

Subject No. 3 received by intravenous injection 20 c.c. of Mulford's polyvalent (types I, II and III) antipneumococcal serum. The injection occupied 10 minutes.

Blood was drawn by venipuncture from each of these three persons immediately before the injection of serum and at the following times after the injection—1 hour, 3 hours, 6 hours, 10½ hours, 24 hours and 36 hours. The bactericidal action of each specimen of defibrinated blood was tested against the three types of pneumococci by the method described. The results of Experiment 1, recorded in Table I, showed that the administration of Lederle's serum was followed by a large and well-maintained increase of bactericidal power against type I and type II pneumococcus, and that the bactericidal power of the blood against type III showed no variation from that before injection greater than one dilution of the pneumococcus culture—a change which is obviously very small compared with the increase seen against types

I and II organisms, and may be considered within the range of experimental error.

In Experiment 2 the result of injecting 20 c.c. of Burroughs Wellcome type I antipneumococcal serum was to produce an increase of bactericidal action against type I organism to the extent of two centimal dilutions. With type II pneumococcus the bactericidal power was only once greater after serum

TABLE I.—*Subject* 1: *Bactericidal Test.*

	Dilutions of pneumococcus culture.							
Pneumococcus type I:	10^{-2}	10^{-4}	10^{-6}	10^{-8}	10^{-10}	10^{-12}	10^{-14}	10^{-16}
Blood before injection . .	4	4	4	4	4	4	4	0*
,, 1 hour after injection .	4	4	4	0	0	0	0	0
,, 3 hours ,, ,, .	4	4	0	0	0	0	0	0
,, 6 ,, ,, ,, .	4	4	0	0	0	0	0	0
,, 10½ ,, ,, ,, .	4	4	4	0	0	0	0	0
,, 24 ,, ,, ,, .	4	4	4	0	0	0	0	0
,, 36 ,, ,, ,, .	4	4	4	4	0	0	0	0
Pneumococcus type II:								
Blood before injection . .	4	4	4	4	4	4	0	0
,, 1 hour after injection .	4	4	4	0	0	0	0	0
,, 3 hours ,, ,, .	4	4	4	0	0	0	0	0
,, 6 ,, ,, ,, .	4	4	4	4	0	0	0	0
,, 10½ ,, ,, ,, .	4	4	4	0	0	0	0	0
,, 24 ,, ,, ,, .	4	4	4	0	0	0	0	0
,, 36 ,, ,, ,, .	4	4	4	4	0	0	0	0
Pneumococcus type III:								
Blood before injection . .	4	4	4	4	4	4	4	0
,, 1 hour after injection .	4	4	4	4	4	4	4	0
,, 3 hours ,, ,, .	4	4	4	4	4	4	4	0
,, 6 ,, ,, ,, .	4	4	4	4	4	4	0	0
,, 10½ ,, ,, ,, .	4	4	4	4	4	4	0	0
,, 24 ,, ,, ,, .	4	4	4	4	4	4	0	0
,, 36 ,, ,, ,, .	4	4	4	4	4	4	4	0

* The figures 4, 3, 2, 1 or 0 indicate the relative amount of growth which occurred when a loopful of the mixture of blood and emulsion of organisms was inoculated on a plate of heated blood-agar medium.

injection than before—at 24 hours—and was otherwise equal to the action shown before injection, or less than it to the extent of one dilution. Against type III the bactericidal power was never greater than before injection of the serum.

In Experiment 3 the administration of 20 c.c. of Mulford's polyvalent serum produced no decisive effect on the bactericidal power against types I and III organisms, but there was an increase to the extent of two centimal dilutions against pneumococci of type II.

It must be noted that no fair comparison can be made on the basis of these results between the Lederle serum and the other two preparations because, although a full therapeutic dose (10 c.c.) of the former serum was administered, only about one-fifth (20 c.c.) of the usual dose advised for use in a case of lobar pneumonia was injected in either of the two latter. It was considered undesirable to give the full dose of 100 c.c. of horse-serum to persons not suffering from pneumococcal infection.

Experiment 4.

The subject was a male, aged 33 years. He was given an intravenous injection of 10 c.c. of the same batch of Lederle's serum as used in Experiment 1. The conjunctival test was negative, and the time occupied by injection ten minutes. Blood was withdrawn and defibrinated at the following times after the injection—1½ hours, 3 hours, 6½ hours, 24 hours, 35 hours.

The specimen of blood drawn before injection of anti-serum allowed survival and multiplication of the organisms of each type (I, II and III) in all centimal dilutions up to and including 10^{-12}. The blood withdrawn one and a half hours after injection was much more active in preventing the survival of organisms of types I and II, 10^{-6} and 10^{-8} respectively being the highest dilutions which yielded growth when the mixtures of blood and organisms were plated out after incubation, but in the case of the type III pneumococcus there was no difference in bactericidal action between the blood taken before the injection of serum and the first specimen withdrawn afterwards. The results with the remaining specimens of blood showed that the bactericidal power against type II organism had fallen to the normal level by the time that the last specimen was taken (35 hours after the injection of serum), but in the case of type I pneumococcus all the specimens taken subsequent to serum injection showed greater bactericidal power than the blood removed before injection, although there was a progressive decrease towards the initial value.

Against type III pneumococcus the bactericidal power of the blood was the same 1½ hours, 3 hours and 35 hours after injection, and the differences recorded in the 6½- and 24-hour specimens were of only one dilution, one greater and one less than the normal. The practical constancy of the action of the blood against this type of pneumococcus constitutes a form of control on the results obtained with the other two types.

Leucocyte counts made at the same time as the venipuncture was performed showed that during the 35 hours the subject was under observation the number of white blood-corpuscles varied within the range 5700 to 7100 per c.mm.

Experiment 5.

A male, aged 41 years, was given, by intravenous injection, 5 c.c. of a preparation of Lederle's types I and II serum, which was double the concentration of that previously used, so that the same number of "units of antibodies" (according to the estimation of the makers of the serum) was administered to this subject as to Nos. 1 and 4. The time taken for the injection of the serum was 10 minutes.

Blood was withdrawn immediately before the serum was injected and at the following times after it—1 hour, 3½ hours, 11 hours, 25 hours and 30 hours.

In this case the test was carried out with pneumococci of only types I and II, and the results showed that the injection of the serum was followed by an increase of bactericidal power of the blood to a degree represented at its maximum by five decimal dilutions of the pneumococcus culture, and gradually becoming less towards the end of the period under observation. With type II organism the maximum increase was equal to that seen in the case of type I, but it occurred sooner than and was not so well maintained as the maximum action against type I.

Experiment 6.

A male, aged 43 years, was given an injection similar to Subject 5—5 c.c. of the doubly concentrated Lederle serum.

Specimens of blood were withdrawn before injection of the serum and 2¼, 8, 24, 32½, 48 and 72 hours after it. A great increase of action against type I organism was found, represented at the maximum by six decimal dilutions of the organism, and this had not passed off entirely at 72 hours after injection of the serum. The normal action of the subject's blood against type II organism was high and the increase after injection was only of two dilutions of the organismal suspension, and had practically entirely passed off in the last specimen. Against type III organism the action of the blood never rose higher than that of preventing the growth of the organisms in the 10^{-5} dilution.

ACTION OF THE BLOOD-SERUM IN PROTECTING MICE AGAINST PNEUMOCOCCAL INFECTION.

In the case of each of the specimens of blood withdrawn from the six subjects in this experiment sufficient was taken for the carrying out of the bactericidal test already described, and also for performing a test on the power of the serum of the subject to protect mice against infection by type I pneumococci when serum and organisms were injected together into the peritoneal cavity.

Method.—The serum was separated from the corpuscles by allowing the blood to coagulate and separating and removing the clot or by centrifuging the blood, or by a combination of both methods. Decimal dilutions of type I pneumococcus up to 1 in 10 millions or higher were made in saline in the case of the test with the sera from subjects 1–4 inclusive, and in broth for those from subjects 5 and 6. Mixtures of serum and organisms were then made so that each specimen of serum was mixed with each dilution of organism. For the specimens from Subject No. 1 only one mouse was injected with each mixture, but two mice were injected with each in the case of sera from the other five people. The amount of the injection was 1 c.c., and it was made into the peritoneal cavity. In the case of the first four subjects, each cubic centimetre of mixture was made up of 0·5 c.c. of serum and 0·2 c.c. of pneumococcal culture with the addition of 0·3 c.c. of saline; with the specimens from Subjects 5 and 6, 0·2 c.c. of serum and 0·8 c.c. of pneumococcal culture were mixed without addition of saline. Mice were also injected with the pneumococcal dilutions alone.

The results of the tests with the sera from Subject 1 are shown in Table II, in which the figures indicate the number of mice which died after the injection of each mixture, and this table is illustrative of the results obtained with the

sera from the other five subjects. The serum removed before the subject received therapeutic antiserum did not show protective action in any case except Subject 3; in this case two mice survived the injection of the 10^{-8} dilution of culture with the addition of serum, while only one of two survived without it. The sera withdrawn after the injection of Lederle's antiserum in Subjects 1, 4, 5 and 6 showed a very great and well-sustained increase of protective action. In the maximum degree in which it was demonstrated it saved mice from a dose of pneumococci ten thousand times as large as the smallest ascertained fatal amount, and the longest time during which the protective action was demonstrated was 72 hours. The protective action of the serum withdrawn from Subjects 2 and 3 did not show an increase after the injection of the therapeutic serum into the subject in any way comparable with the increases seen in the other four subjects.

TABLE II.—*Subject* 1: *Test of Protective Power of Serum.*

Pneumococcus type I:	Dilutions of pneumococcus culture.				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
No serum (control) . .	..	..	2	2	1*
Serum before injection . .	..	2	2	2	1
,, 1 hour after injection .	0	0	0	0	..
,, 6 hours ,, ,, .	0	0	0	..	..
,, $10\frac{1}{2}$,, ,, ,, .	0	0	0	..	..
,, 24 ,, ,, ,, .	0	0	0	..	..
,, 36 ,, ,, ,, .	0	0	0	0	..

* The figures 2, 1 or 0 indicate the number of mice which survived the injection of pneumococci with or without the addition of serum.

DISCUSSION.

The results of these experiments show that the intravenous injection of concentrated antipneumococcus serum can increase for as long as 72 hours the bactericidal power of the blood against pneumococci homologous to the serum used. This in itself gives hope that the use of such serum might be valuable in the treatment of lobar pneumonia, but during the infection a partial antigen of the pneumococcus—the specific soluble substance—is present in the body in large amounts, and it has been demonstrated that it decreases the bactericidal power of blood (Sia, 1926; Alston, Galbraith and Stewart, 1930), probably on account of its well-known action of uniting with antipneumococcal antibodies. The presence of the specific soluble substance in increasing amounts during the course of the infection may partly account for the decrease in effect of serum administration as the disease progresses. This decrease is emphasized by those who have reported on the use of serum in human disease, and it was demonstrated by Goodner (1928) when serum was used to check pneumococcal infection in rabbits. The results recording the action of the serum of the subjects experimented upon in protecting mice against high multiples of the average lethal dose of pneumococcus corroborate the *in vitro* bactericidal results, and

emphasize the power of serum to aid in destroying bacteria in the body at the earlier stages of infection.

SUMMARY AND CONCLUSIONS.

1. The bactericidal power of the blood and the protective action of the serum may be greatly increased for at least 72 hours as regards types I and II pneumococcus by the injection into a " normal " uninfected person of a therapeutic dose of a preparation of serum containing immune bodies against those two types of pneumococcus.

2. The validity of the results is checked by the lack of change in the bactericidal power of the blood of the subjects against the type III organism, and the smaller or negligible increase in bactericidal power of blood or protective action of blood-serum when the injection given was a fraction of a therapeutic dose of other preparations of immune sera. The results with these latter preparations are included for purposes of such control.

We desire to offer our thanks to Prof. T. J. Mackie for his interest and guidance in this work.

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